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POULTRY SCIENCE

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THE EFFECT OF VARIOUS CONCENTRATIONS OF NICOTINE IN TOBACCO ON THE GROWTH AND DEVELOPMENT OF FOWLS

I. A STUDY OF THE NICOTINE TOLERANCE OF GROWING CHICKS*

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(Received for Publication 8-5-30)

The feeding of tobacco for the control of intestinal parasites in fowls has long been practiced. This is true, especially, for round worms (*Ascaridia lineata*) in chicks. As ordinarily practiced, however, the feeding of tobacco in the ration appears to interfere with the normal metabolism of the chicks. Considerable attention, therefore, has been given to the quantity of tobacco fed and the duration of feeding. Beach and Freeborn¹, for example, have recommended that a ration containing two per cent of tobacco of one and one-half percent nicotine be fed for periods of three or four weeks, and then discontinued. Recently, however, these investigators² have called attention to the fact that the tobacco appears to make the ration unpalatable and that the birds that are most seriously affected with internal parasites may not partake of it to any considerable extent. They now recommend the feeding of a nicotine sulfate preparation but find that this practice is not entirely satisfactory, as it may prove toxic to the birds themselves.

* Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 510.

¹ BEACH, J. R., AND FREEBORN, S. B. Cal. Agr. Exp. Sta. Circular No. 251, 1922.

² BEACH, J. R., AND FREEBORN, S. B. Cal. Agr. Extension Circular No. 8, 1927.

Phillips, Carr, and Kennard³ found that ground tobacco added to the ration of growing chicks prevents their normal growth and causes an undue stimulation, as evidenced by a nervous condition of the individual chicks. They found, however, less nitrogen in the feces of the chicks fed tobacco as compared to those fed a tobacco-free ration, a fact which may signify a better utilization of proteins as a result of tobacco feeding.

The problem of feeding tobacco to chicks merits further study. It is fairly well established that nicotine is the active vermifugal agent of tobacco. If, therefore, a strain of tobacco of high nicotine content is used, less total tobacco would be required to maintain a given concentration of nicotine in the feed than of tobacco possessing a low nicotine content. In this way there is prevented an undue concentration in the ration of tobacco compounds other than nicotine, which may prove undesirable. This is a question of considerable importance. In our experimental work an effort was made to ascertain whether chicks could tolerate more nicotine in high-nicotine tobacco than in low-nicotine tobacco, as measured by the effect on growth and development.

Living organisms including those of a high order, are, as a rule, quite susceptible to the action of nicotine. On the other hand, through the rational use of this compound, it is possible for a higher organism to develop a marked degree of tolerance. In our experimental work increasing quantities of tobacco were fed in order that a certain degree of tolerance could be attained; also in order to maintain a maximum quantity of nicotine according to the treatment for a period beyond which, according to Ackert⁴, there is very little danger of infestation.

EXPERIMENTAL

Five groups of eighteen 1-day old Single Comb White Leghorn chicks were placed in battery brooders having wire-screened floors. Group I was used as a control and received no tobacco supplement. Groups II, III, IV and V received different quantities of finely ground tobacco (5 per cent nicotine) which was thoroughly mixed with their ration. All groups were

³ PHILLIPS, A. G., CARR, R. H., AND KENNARD, D. C. *Jour. Agr. Res.*, Vol. 20, p. 869, 1921.

⁴ ACKERT, J. E. Private communication. 1930.

kept on Ration 1 for a period of 8 weeks, when they were given Ration 2 for the remainder of the feeding trial, 10 weeks.

At the same time, an effort was made to study the effect of tobacco feeding on older chicks. Five groups of eight 12-week old chicks were confined in fattening batteries for 12 weeks. Group VI served as a check. The quantity of tobacco (5 per cent nicotine) fed Groups VII, VIII, IX and X corresponded to the quantities fed Groups II, III, IV, and V in respective order. These birds were fed on Ration 2 throughout the entire period.

The composition of the two rations is given in Table I.

TABLE I.—COMPOSITION OF THE RATIONS USED.

Constituents	Ration 1 pounds	Ration 2 pounds
Yellow corn meal	40	40
Wheat bran	15	15
Flour, wheat middlings	15	15
Alfalfa leaf meal	10	10
Dry buttermilk	10	10
Fish meal	5	5
Meat scrap	5	5
Steamed bone meal	2	2
Sodium chloride (NaCl)	1	1
Cod liver oil	1	1
Ground oats	0	10
Total	104	114

The percentages of ground tobacco (5 per cent nicotine) and nicotine in the feed of the different groups are shown in Table II.

TABLE II.—THE CONCENTRATION OF TOBACCO AND NICOTINE IN THE RATIONS USED

Group No.	First Week		Second Week		Third and Remaining Weeks	
	Tobacco per cent	Nicotine per cent	Tobacco per cent	Nicotine per cent	Tobacco per cent	Nicotine per cent
I, VI	0	0	0	0	0	0
II, VII	.05	0.0025	.1	0.005	.2	0.01
III, VIII	.10	0.0050	.2	0.020	.4	0.02
IV, IX	.20	0.0100	.4	0.040	.8	0.04
V, X	.30	0.015	.6	0.030	1.2	0.06

Table III shows the quantity of feed and tobacco consumed by the young chicks for a period of 18 weeks.

TABLE III.—THE QUANTITY OF FEED AND TOBACCO CONSUMED PER CHICK PER WEEK.

Period Weeks	Group I		Group II		Group III		Group IV		Group V	
	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.
1	55.5	00.0	52.8	.0264	55.5	.0555	55.5	.1110	61.1	.1833
2	61.1	00.0	63.9	.0639	63.9	.1278	69.4	.2676	63.9	.3834
3	107.0	00.0	95.8	.1916	108.3	.4532	116.7	.9336	111.0	1.3320
4	127.8	00.0	118.1	.2362	117.8	.4712	116.7	.9336	111.0	1.3320
5	150.0	00.0	161.1	.3622	170.6	.6824	156.9	1.2552	159.7	1.9164
6	222.2	00.0	235.3	.4706	229.4	.9176	241.7	1.9336	205.6	2.4672
7	241.7	00.0	261.8	.5236	252.9	1.0116	216.7	1.7336	252.9	3.0348
8	280.9	00.0	272.1	.5442	276.5	1.1060	283.3	2.2664	250.0	3.0000
9	344.1	00.0	329.4	.6588	355.9	1.4236	350.0	2.8000	341.7	4.1004
10	364.7	00.0	394.1	.7882	394.1	1.5764	383.3	3.0664	419.4	5.0328
11	412.2	00.0	441.2	.8824	397.1	1.5884	369.4	2.9552	416.7	5.0004
12	343.8	00.0	352.9	.7058	335.3	1.3412	411.1	3.2888	400.0	4.8000
13	445.3	00.0	470.6	.9412	435.3	1.7412	480.6	3.8448	505.9	6.0708
14	488.3	00.0	479.4	.9588	452.4	1.8096	494.4	3.9552	526.5	6.3180
15	493.3	00.0	458.8	.9176	441.2	1.7648	483.3	3.8664	529.4	6.3528
16	500.0	00.0	482.4	.9648	441.2	1.7648	491.7	3.9336	547.1	6.5652
17	560.0	00.0	517.7	1.0354	482.4	1.9296	488.9	3.9112	538.2	6.4584
18	586.7	00.0	511.8	1.0236	482.4	1.9296	505.6	4.0448	567.6	6.8112

TABLE IV.—THE QUANTITY OF FEED AND TOBACCO CONSUMED PER CHICK PER WEEK.
MATURE BIRDS

Period Weeks	Group VI		Group VII		Group VIII		Group IX		Group X	
	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.	Feed gms.	Tobacco gms.
1	400.0	00.0	362.5	.1813	375.0	.3750	337.5	.6750	343.8	1.0314
2	462.5	00.0	437.5	.4375	425.0	.8500	462.5	1.8500	375.0	2.2500
3	425.0	00.0	350.0	.7000	346.9	1.3876	362.5	2.9000	375.0	4.5000
4	481.3	00.0	418.8	.8376	446.9	1.7876	453.1	3.6248	468.8	5.6256
5	512.3	00.0	512.5	1.0250	553.1	2.2124	528.1	4.2248	543.8	6.5256
6	493.8	00.0	487.5	.9750	450.0	1.8000	468.8	3.7504	500.0	6.0000
7	537.5	00.0	693.8	1.3876	625.0	2.5000	581.3	4.6504	562.5	6.7500
8	506.3	00.0	625.0	1.2500	606.3	2.4252	516.8	4.1344	556.3	6.6756
9	612.5	00.0	650.0	1.3000	706.3	2.8252	656.3	5.2504	625.0	7.5000
10	557.1	00.0	516.8	1.0336	581.3	2.3252	581.3	4.6504	587.5	7.0500
11	571.4	00.0	662.5	1.3250	781.3	3.1252	593.8	4.7504	606.3	7.2756
12	550.0	00.0	584.8	1.1696	692.5	2.7700	560.0	4.4800	602.5	7.2300

Chart I shows the growth curves of both males and females of Groups I, II, III, IV, and V. Those groups receiving the tobacco showed better growth than the Control Group. During this trial three birds died in the Control Group, one in Group II and two in Group III. All of the chicks in Groups IV and V were living at the end of the experiment.

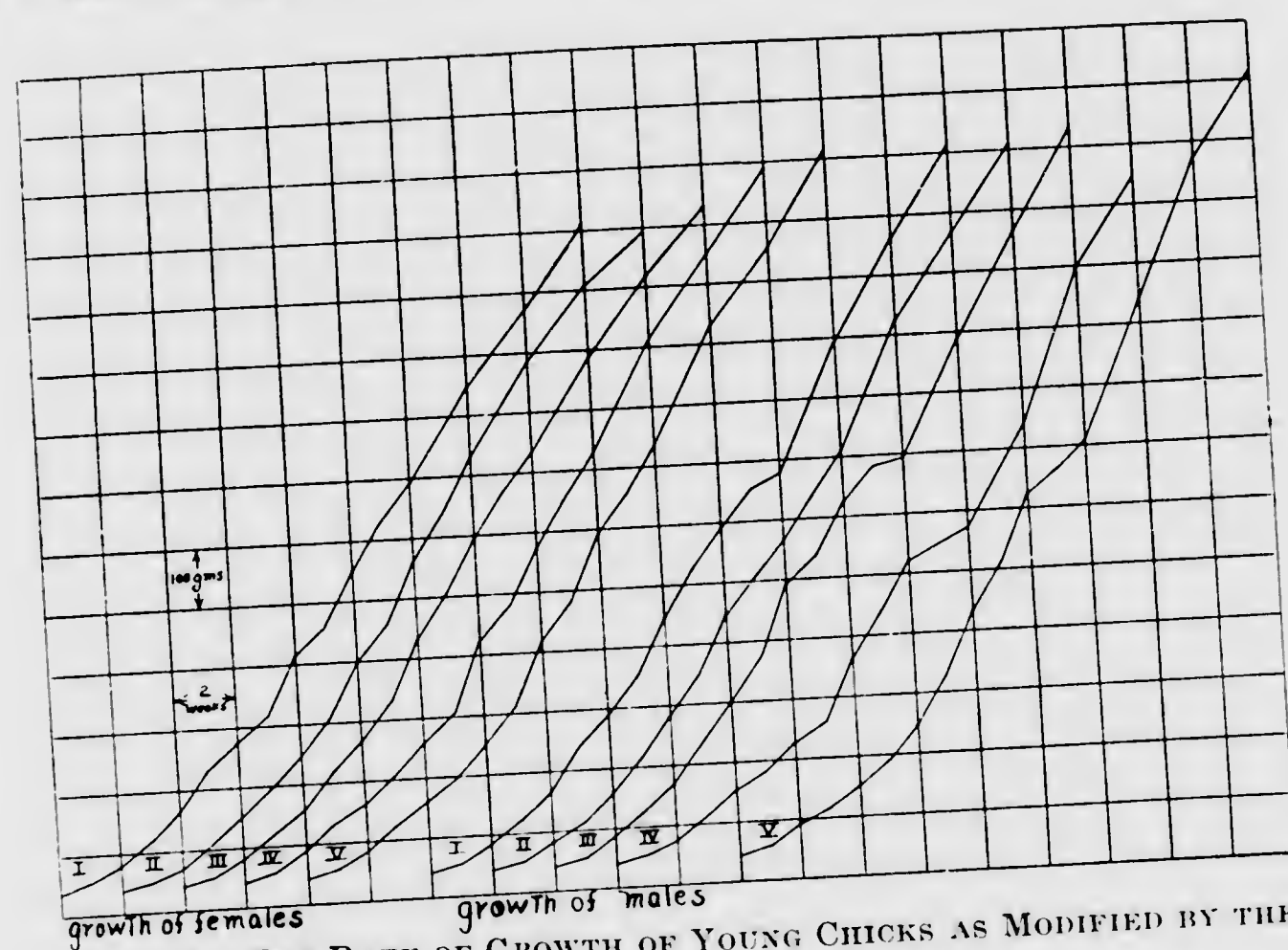


CHART I.—THE RATE OF GROWTH OF YOUNG CHICKS AS MODIFIED BY THE TOBACCO CONTENT OF THE RATION.

Chart II shows the growth curves of both males and females of Groups VI, VII, VIII, IX, and X. While the birds of the different groups were not as uniform as the preceding groups, nevertheless it is evident that the tobacco supplement to the ration did not restrict the growth of the birds. There was no mortality in any of the groups of this series.

Postmortem studies were conducted on all birds and no case was noted where tobacco feeding had a harmful effect. Experiments now in progress in which high nicotine levels are maintained by tobacco of 0.86 per cent nicotine content, show results that are decidedly harmful. These results will be published later.

It is the intention of the authors to artificially infest birds

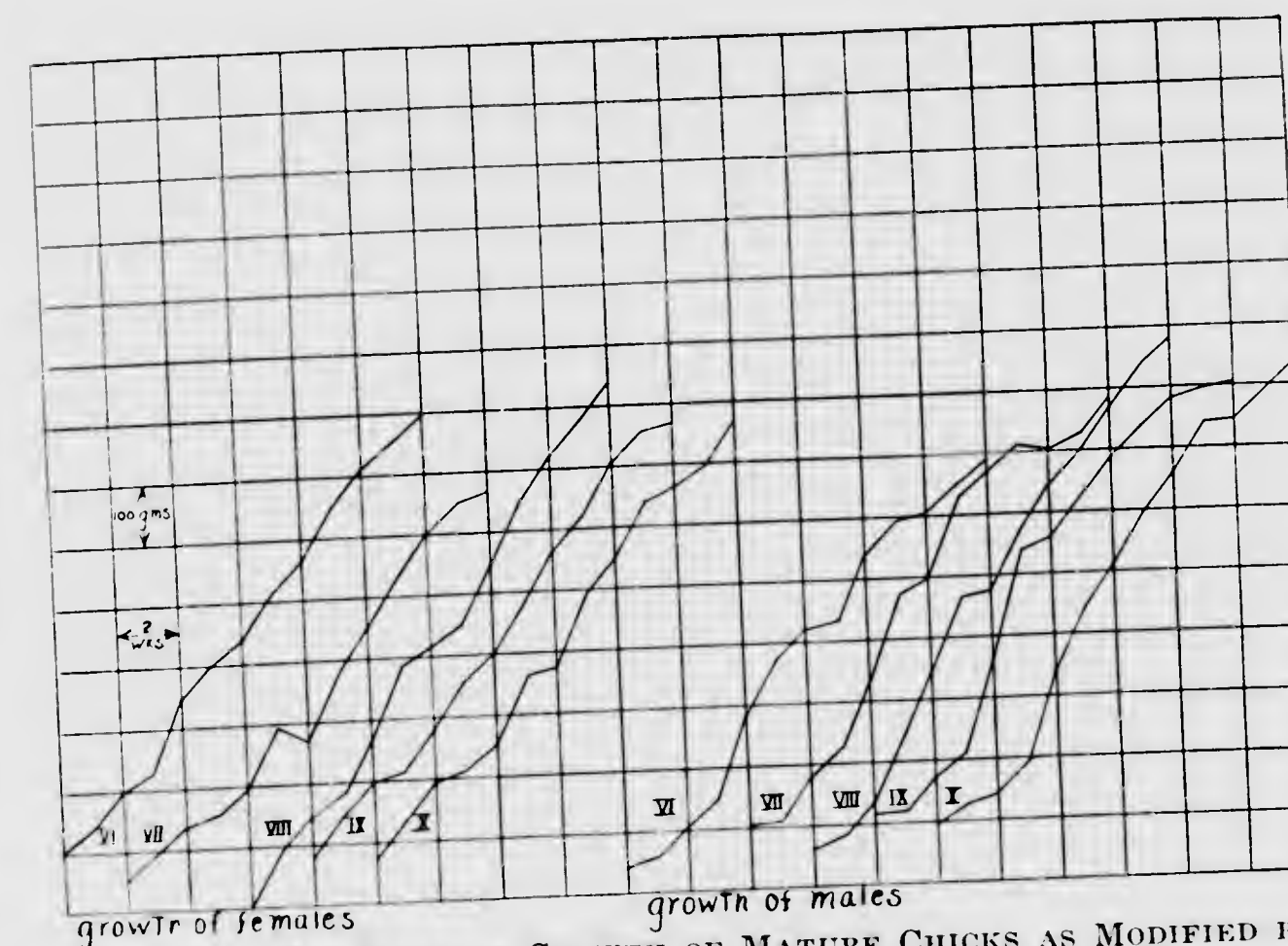


CHART II.—THE RATE OF GROWTH OF MATURE CHICKS AS MODIFIED BY THE TOBACCO CONTENT OF THE RATION.

with *Ascaridia lineata* and to learn, by post-mortem studies, at which level of nicotine feeding infestation is prevented. It is quite probable that with relatively large quantities of nicotine passing continually through the intestinal tract from hatching date to maturity, birds may be raised on worm-infested ground without danger of infestation.

CONCLUSIONS

These studies show that chicks can tolerate larger quantities of nicotine in tobacco than has hitherto been recommended, if a high nicotine strain of tobacco is used for this purpose.

Reprinted from PLANT PHYSIOLOGY, 6: 177-182, 1931.

COMPOSITION AND QUALITY OF PENNSYLVANIA CIGAR-LEAF TOBACCO AS RELATED TO FERTILIZER TREATMENT^{1, 2}

D. E. HALEY, J. B. LONGENECKER AND OTTO OLSON

The quality of the smoke produced during the normal combustion of a cigar is largely dependent on the chemical composition of the tobacco. The chemical composition of the tobacco may be influenced by many factors, among which is the fertilizer treatment employed by the grower. In order to obtain the best quality cigar-leaf tobacco, it is essential to recognize the requirements and limitations of the growing plant, to insure the presence of the materials deemed necessary, and to guard against those materials that are not only unnecessary but even harmful, so far as quality production is concerned. The individual effects of a fertilizer treatment, however, may be considerably influenced by many environmental conditions, among which is the inherent fertility of the soil itself. For example, the soil of the experimental tobacco plots at Ephrata, Lancaster County, Pennsylvania is extremely high in calcium and quite low in available potassium. This may not hold true for tobacco soils in other sections of the county; hence the optimum fertilizer treatment for the experimental plots may not hold true for other soils in the county.

In former publications (1, 2) we have recognized the fact that for quality production of tobacco on our experimental plots we must equalize, if possible, the calcium and potassium content of the plants. In 1928 we planned an experiment having this objective.

Plan of the experiment

Avoiding the use of lime, but not taking into account those factors which might influence the availability of soil calcium, we planned an experiment making potash the most important variable. Ten plots were used, each one-thirtieth of an acre in area. The crop previously grown on these plots was red clover. Five of the plots received manure, with or without additions of fertilizer. The remainder received individual fertilizer treatments, but no manure. The treatments are given in table I.

At the end of the growing season twenty plants from each plot were air-cured. At the end of this period they were dried at room temperature. When dry, the webs of the leaves were ground and kept in sealed receptacles for analysis.

¹ Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper no. 505.

² This investigation was conducted in cooperation with Dr. W. W. GARNER, of the U. S. Bureau of Plant Industry, Office of Plant Nutrition and Tobacco Investigations, and Professor F. D. GARDNER, Department of Agronomy of the Pennsylvania State College.

TABLE I
FERTILIZER TREATMENTS OF THE EXPERIMENTAL PLOTS
ACRE BASIS

PLOT NO.	MANURE	COTTON-SEED MEAL	PRECIPITATED BONE* PHOSPHATE	NITRATE OF SODA	SULPHATE OF POTASH	CARBONATE OF POTASH	UREA
	<i>tons</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>
A-1 ...	10	400	130	125	200
A-2 ...	10	400	130	125	400
A-3 ...	10	400	130	125	600
A-4 ...	10	400	130	125	310
A-5 ...	10
A-6	600	195	187	300
A-7	600	195	187	600
A-8	600	195	187	900
A-9	600	195	187	465
A-10	375	460	187	465	57

* 36 per cent. P_2O_5 .

The remaining plants were similarly cured; at the end of this period they were fermented for several months. Uniform samples then were taken for analyses, as mentioned above, and a part of the remainder were made into cigars.

Analyses of the samples were made according to methods as described in previous publications (1, 2), with but few modifications. A special effort, however, was made to determine separately the soluble and insoluble portions of the total ash, thus guarding against the possibility of weighing the calcium as the hydrate or carbonate rather than the oxide.

TABLE II
STUDY OF THE CALCIUM, MAGNESIUM, SULPHUR AND POTASSIUM CONTENT OF CURED TOBACCO AS INFLUENCED BY FERTILIZER TREATMENT

PLOT NO.	CAO	MgO	SO ₃	K ₂ O
	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>
A-1	7.95	0.84	1.14	2.57
A-2	7.55	0.75	1.16	3.16
A-3	7.45	0.80	1.32	3.35
A-4	7.76	0.81	1.16	3.21
A-5	7.65	0.96	1.18	1.74
A-6	8.09	0.92	1.40	2.81
A-7	6.97	0.82	1.47	3.26
A-8	7.01	0.85	1.68	3.27
A-9	7.23	0.81	1.05	2.84
A-10	7.67	0.85	1.49	2.23

Analytical results

A study of the calcium, magnesium, sulphur, and potassium of the cured samples was made first; the results are given in table II.

This analysis was followed by a study of the soluble and insoluble ash constituents of both the cured and fermented samples, and their alkalinity; the results are given in tables III and IV.

TABLE III
ASH OF THE CURED AND FERMENTED CIGAR-FILLER TOBACCO AS MODIFIED BY FERTILIZER TREATMENT

PLOT NO.	ASH					
	SOLUBLE		INSOLUBLE		TOTAL	
	CURED	FERMENTED	CURED	FERMENTED	CURED	FERMENTED
	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>
A-1 ...	3.99	5.49	13.47	10.45	17.46	15.94
A-2 ...	4.64	6.01	11.95	10.63	16.59	16.64
A-3 ...	4.76	6.38	12.02	10.10	16.78	16.48
A-4 ...	4.51	5.70	11.89	10.61	16.40	16.31
A-5 ...	2.79	3.77	12.85	10.28	15.64	14.05
A-6 ...	3.57	5.40	12.33	10.67	15.90	16.07
A-7 ...	5.00	4.90	11.48	9.94	16.48	14.84
A-8 ...	5.13	12.74	17.87
A-9 ...	4.23	4.97	12.71	10.53	16.94	15.50
A-10 ...	3.44	5.02	12.74	10.57	16.18	15.59

TABLE IV
ALKALINITY OF THE ASH OF CURED AND FERMENTED TOBACCO

PLOT NO.	ALKALINITY OF ASH					
	SOLUBLE		INSOLUBLE		TOTAL	
	CURED	FERMENTED	CURED	FERMENTED	CURED	FERMENTED
	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
A-1 ...	46.7	43.6	309.6	318.7	356.3	362.3
A-2 ...	45.7	48.3	286.5	310.6	332.5	358.9
A-3 ...	36.6	47.0	292.5	300.5	329.1	347.5
A-4 ...	38.5	49.6	287.0	312.6	325.5	362.2
A-5 ...	28.1	28.2	287.0	307.9	315.1	336.1
A-6 ...	25.1	40.2	332.4	335.4	357.5	375.6
A-7 ...	37.3	30.2	296.0	293.2	333.3	323.4
A-8*	30.1	300.0	330.1
A-9 ...	35.4	41.9	309.1	305.9	344.5	347.8
A-10 ...	31.3	37.5	332.3	317.0	363.6	354.5

* The sample of fermented material from this plot was not received.

The nitrogen-carbon ratio of the cured and fermented samples then was ascertained (table V) and the results of smoking the experimental cigars are given in table VI.

TABLE V
NITROGEN-CARBON RATIO OF CURED AND FERMENTED TOBACCO

PLOT NO.	CARBON		NITROGEN		N-C RATIO	
	CURED	FERMENTED	CURED	FERMENTED	CURED	FERMENTED
	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>		
A-1	40.25	40.74	4.42	5.17	1: 9.1	1: 7.9
A-2	42.06	40.01	4.38	5.00	1: 9.6	1: 8.2
A-3	40.96	41.16	4.29	4.81	1: 9.5	1: 8.6
A-4	42.05	41.62	4.80	4.71	1: 8.8	1: 8.8
A-5	41.86	41.17	4.47	4.95	1: 9.4	1: 8.3
A-6	40.92	41.07	4.51	4.93	1: 9.1	1: 8.3
A-7	40.95	41.56	4.57	5.14	1: 9.0	1: 8.1
A-8	42.11	4.44	1: 9.5
A-9	40.07	41.36	4.51	5.18	1: 8.9	1: 8.0
A-10	42.24	42.03	4.24	4.45	1: 10	1: 9.4

Discussion of results

Analyses of the cured samples showed that it was not possible to materially decrease the calcium or increase the potassium content by the fertilizer treatments employed; this is shown in table II. Analyses of the fermented samples, however, as shown in table III, indicate a more narrow potassium-calcium ratio. According to table II, the sulphur content was not influenced by the large quantity of sulphate of potash additions. These results indicate that the solution of the problem may not be reached by potash applications alone. More attention must be given to those factors which have to do with the availability of soil calcium. In this connection the advisability of plowing under a crop of clover, which is a heavy calcium feeder and maintains a high nitrogen content of the soil, is questioned.

The data in table III show a marked decrease in soluble ash of the fermented, as compared with the cured samples. Probably this is due to the loss of such material in the handling of the tobacco. Considerable quantities of soil and sand may adhere to the leaves of the harvested plants, owing to a gummy covering of the leaves, and even may persist over the period of air-curing. As this gummy material disappears during fermentation, there is a chance for a noticeable loss of inorganic material in this process. At the same time, there is a possibility that an equilibrium is established between the web and the midrib or stem during fermentation, resulting in a loss of calcium from the web to the midrib or stem and an increase of substances, such as potassium, in the web at the expense of these portions.

TABLE VI
SMOKING QUALITIES OF CIGARS MADE FROM THE 1928 CROP
THREE-YEAR ROTATION*

SAMPLE NO.	BURN	FIRE-HOLDING CAPACITY	COLOR OF ASH	COHERENCE OF ASH	AROMA	TASTE	TOTAL**
	<i>points</i>	<i>points</i>	<i>points</i>	<i>points</i>	<i>points</i>	<i>points</i>	<i>points</i>
A-1	18	10	8	8	15	15	74
A-2	17	10	8	6	18	15	74
A-3	18	10	6	8	18	15	75
A-4	19	10	8	8	18	15	78
A-5	15	5	8	6	10	12	56
A-6	18	10	6	8	16	14	72
A-7	18	10	6	8	16	14	72
A-8	18	10	8	8	18	16	78
A-9	16	7	6	8	16	14	67
A-10	16	10	5	8	16	14	69

- * A-1 Coherent. Light gray ash. Slight char. Holds fire 6-7 minutes.
A-2 Flakes slightly. Light ash. Slight char. Holds fire 6-7 minutes.
A-3 Coherent. Medium gray ash. Slight char. Holds fire 6-7 minutes.
A-4 Coherent. Light ash. Slight char. Holds fire 6-7 minutes.
A-5 Fairly coherent. Mottled ash. Chars badly. Holds fire less than 5 minutes.
A-6 Coherent. Medium gray ash. Chars slightly. Holds fire 6-7 minutes.
A-7 Coherent. Medium gray ash. Chars slightly. Holds fire 6-7 minutes.
A-8 Coherent. Light gray ash. Chars very slightly. Holds fire 6-7 minutes.
A-9 Coherent. Medium gray ash. Chars. Holds fire 5-6 minutes.
A-10 Coherent. Dark ash. Chars. Holds fire 6-7 minutes.

** A perfect score would have been as follows:

Burn	20 points
Fire-holding capacity	10 points
Color of ash	10 points
Coherence of ash	10 points
Aroma	25 points
Taste	25 points

There has been a material increase, in most instances, in the soluble ash of the web during fermentation; these differences in percentages cannot be accounted for on the basis of a loss of organic matter during fermentation. If this is correct, then the fermentation of cigar-leaf tobacco has a significance not usually taken into consideration. In order to correctly interpret the data in tables III and IV, however, the total loss of ash constituents and organic matter should be known.

The data in table V show, with one exception, a narrowing of the nitrogen-carbon ratio in the fermentation process. They further show that there must have been a relatively small amount of nitrogen lost during the fer-

mentation process. It must be remembered, however, that the fermentation was not sufficiently prolonged to insure the best quality of tobacco. The carbon content of the cured and fermented samples is, on the whole, quite uniform.

Table VI shows that cigars made from the tobacco of plot A-5 were inferior to the others. On the whole, however, the smoking tests were unsatisfactory. We believe that the lack of sufficient fermentation and aging were of overshadowing importance. All cigars tested showed a relatively high chlorophyll content.* In order to study the effect of fertilizer treatments on tobacco by the score-card system employed, it is desirable that the tobacco be fermented and aged for a much longer period of time before being made into cigars for testing.

Conclusions

The results of these investigations appear to warrant the following conclusions:

1. The fertilizer treatments employed did not materially alter the ratio of potassium to calcium in the cured leaves.
2. Increasing the quantity of sulphate of potash in the fertilizer treatments does not result in increasing the quantity of sulphur in the leaves.
3. During the process of fermentation there is an apparent increase of soluble ash constituents in the leaves.
4. There is a narrowing of the nitrogen-carbon ratio during fermentation, but very little, if any, loss of nitrogen.
5. The effect of the fertilizer treatment on the burning qualities of the cigars made from the differently treated tobaccos, is overshadowed by insufficient fermentation and aging. Qualitative tests of these cigars showed a relatively high concentration of chlorophyll, which is not only undesirable itself, but also indicates insufficient fermentation.

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* Chlorophyll, however, may be found in many of the cigars now on the market.

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A STUDY OF THE AMMONIA CONTENT OF CIGAR SMOKE^{1, 2}

D. E. HALEY, C. O. JENSEN AND OTTO OLSON

(WITH ONE FIGURE)

The number of cigars manufactured in the United States for the year 1913 exceeded 8,500,000,000, while in 1928 the number produced was about 7,000,000,000. These figures indicate a noticeable decrease in demand. Probably the chief cause for this is the increasing popularity of cigarettes. However, the quality of the cigars now on the market may be a factor of considerable importance. The curing, fermentation, and aging processes have a great deal to do with the development of a pleasant aroma, lack of harshness, and a mild physiological effect when smoked, but the field treatment of the tobacco itself may be of overshadowing importance.

Pennsylvania ranks high as a producer of cigar-leaf tobacco. The Pennsylvania Agricultural Experiment Station and the United States Department of Agriculture have been interested for a number of years in the factors that affect quality production. Various field treatments have been employed at the Tobacco Experiment Plots at Ephrata. Each year a considerable quantity of the tobacco produced is fermented and made into cigars for testing purposes. As a rule, the cigars are made from tobacco less than a year after the crop is harvested. Because of insufficient aging, these cigars usually produce a harsh unpleasant smoke. The burn, coherence of ash, and other qualities may, however, be studied satisfactorily.

The physiological effect and other undesirable qualities of cigar smoke have been attributed, in large measure, to the nicotine content, although cigar smoke is known to contain ammonia, aldehydes, amines, organic acids, carbon monoxide, hydrocarbons, hydrogen sulphide, hydrogen cyanide, pyridine, and many other substances. From a physiological standpoint, ammonia is an important constituent in that it may interfere with the normal action of the heart and produce other complications if present in the smoke in sufficient concentrations.

Very little has been reported on the ammonia content of cigar smoke, especially for cigars made from tobacco of known history. For this reason it was decided to make a study of this constituent in the smoke of cigars made from the experimental tobaccos.

¹ Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper no. 506.

² This investigation was conducted in cooperation with Dr. W. W. GARNER, of the U. S. Bureau of Plant Industry, Office of Plant Nutrition and Tobacco Investigations, and Professor F. D. GARDNER, Department of Agronomy of the Pennsylvania State College.

Experimental

The test cigars were made wholly of tobacco grown on 10 separate plots which received fertilizer treatments according to the plan given in a previous paper (5). An intermittent smoking apparatus was used. Somewhat similar methods have been employed by others. JENKINS (6) used an apparatus in which suction was secured by means of an aspirator which filled by a continuous inflow of water and emptied at regular intervals by means of a siphon. GARNER (4) retained the essential features of this apparatus, but modified it so that several cigars could be smoked simultaneously. GARNER's apparatus, however, was devised for work pertaining to the burning qualities of the cigar rather than to the chemistry of the smoke. The duration of each puff was 10 seconds; the interval between puffs was 30 seconds. WILEY (9) describes a similar apparatus. ASHERSON (1) used an aspirator which evidently was turned on and off by hand, so as to simulate the manner of smoking of the average smoker. BOGEX (2) states that he obtained the necessary suction by the use of a water pump which was turned on and off at regular intervals by an electric solenoid valve, operated by a contact on a Harvard kymograph. An automatic siphon arrangement was tried, but was discontinued as unsatisfactory.

Various methods have been employed for collecting the active constituents of smoke. BOGEX (2) reports that he collected the smoke over water, allowed it to condense for one hour and then analyzed the aqueous solution for ammonia. THOMS (7) employed three jars containing various quantities of 10 per cent. H_2SO_4 in order to remove the basic constituents of the smoke.

Methods of analyses employed by other workers in this field were reviewed. VICKERY and PUCHER (8) developed a method for estimating the ammonia in tobacco and tobacco extracts which is entirely satisfactory for work of this kind. It is based on the observation that nicotine is absorbed by permutit (a synthetic aluminosilicate) only to a very small extent, whereas ammonia may be quantitatively removed from a faintly acid solution by permutit, set free by alkali and determined by Nesslerization. The method which is fully described by VICKERY and PUCHER (8) and which is a modification of FOLIN and BELL's method for the determination of ammonia in urine (3), was used by us and found quite satisfactory.

The smoking apparatus

Some of the apparatus previously used by us proved unsatisfactory. It was felt that an intermittent siphon could be made that would give a regular interval in the suction. An apparatus was devised which proved satisfactory (see fig. 1). It was so regulated that each puff lasted about 6.5 seconds with an interval between puffs of 35 seconds.

A is a suction flask to which continuous suction is applied by means of a laboratory vacuum pump. The amount of suction is regulated by means of a valve. B, C, and D are absorption tubes, each of which holds 25 cc. of 20 per cent. H_2SO_4 . E is a glass cigar holder. F is a tube admitting air to the suction flask A, when the water level in the intermittent siphon G is below the level of the inverted tube F. When the water rises to the level of the inverted funnel, the air supply is cut off and the vacuum created in A draws air through the cigar. When the water reaches the top of the curved tube in G, it siphons out and the tube F is again open, air enters the suction flask A and no air is drawn through the cigar. I is a bottle holding water at a constant level, and fed from the supply bottle J. The rate of flow of water from I is regulated by means of the clamp H.

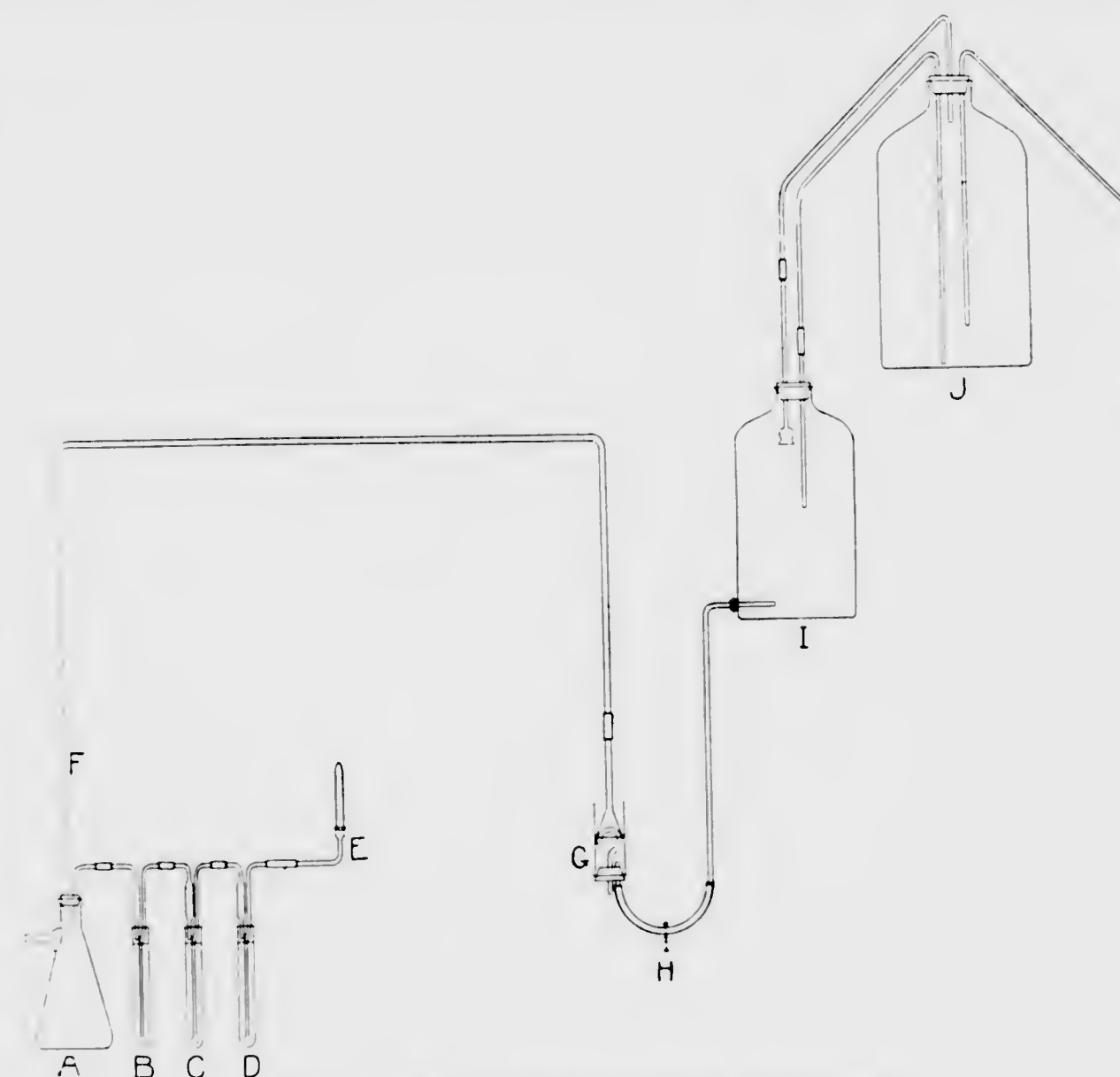


FIG. 1. Intermittent smoking apparatus.

Before smoking, the cigars were kept in a desiccator containing 43 per cent. H_2SO_4 . At 25°C ., according to WILSON (10), this should give an atmosphere having a relative humidity of 50 per cent. The ends of the cigars were cut so that all had the same circumference. Each cigar then was weighed and smoked. The small quantity of tobacco remaining at the

end of the experiment was weighed and subtracted from the original weight. In this way the weight of tobacco smoked was ascertained.

In the preliminary work it was found that 25 cc. of normal H_2SO_4 in the first tube absorbed practically all of the ammonia. Since we desired to determine nicotine, however, we used 20 per cent. H_2SO_4 instead.

Three cigars were smoked before the acid was removed. The total amount of ammonia and its relation to the total nitrogen content of the tobacco then was determined. The results are given in table I.

TABLE I
QUANTITY OF AMMONIA IN SMOKE AS RELATED TO THE TOTAL NITROGEN CONTENT OF THE ORIGINAL TOBACCO FROM WHICH THE CIGARS WERE MADE

CONSTITUENTS	SAMPLES									
	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Total nitrogen	51.7	50.0	48.1	47.1	49.5	49.3	51.4	51.8	44.5
Ammonia in smoke	4.7	5.4	5.5	5.6	5.4	5.4	6.0	4.8	3.5	3.6

The results show that apparently there is no relation between the ammonia content of cigar smoke and the fertilizer treatment received by the tobacco. This is not strange since the fertilizer treatment did not materially affect the nitrogen content of the tobacco. The first seven samples show a close correlation between the total nitrogen content of the tobacco and the quantity of ammonia in the smoke. Representative samples of several commercial cigars made almost wholly of well fermented tobacco showed a smoke of much lower ammonia content.

Summary

1. An apparatus was devised which proved satisfactory for the intermittent smoking of cigars.

2. There was no correlation between the fertilizer treatment of the tobacco and the ammonia content of the smoke. This may not hold true for cigars made of thoroughly fermented tobacco.

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Feed Tobacco Well

Pennsylvania Farmers Find It Pays

By D. E. Haley

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THE system of agriculture followed by the farmers of Lancaster county, Pennsylvania, may well serve as an example of efficiency for many other farming communities in the United States, in that it involves *addition* rather than *subtraction*. In other words, Lancaster county farmers pride themselves in doing all that is humanly possible to keep their soils in a high state of fertility through the liberal use of lime, stable manure, commercial fertilizers, and carefully planned crop rotations which always include legumes.

The original soils were quite fertile and they were selected by the early German settlers for this reason. These settlers, we are told, were representatives of the twenty-fifth successive generation of farmers in their mother country, and thus were well qualified to recognize the crop-producing powers of a soil from a study of the native vegetation. After a farm had been chosen, it was handed down from father to son through successive generations. It has been

our privilege, on several occasions, to visit some of these farms which have been operated by the same family for more than 200 years, and which still are in a high state of fertility. The Ephrata Station is located on one of these farms.

Field work on Pennsylvania tobacco is being conducted at Ephrata, Lancaster county, and Lock Haven, Clinton county. The greater part of this work, however, is conducted at the Ephrata Station under the direction of the Office of Tobacco Investigations, U. S. Bureau of Plant Industry, in cooperation with The Pennsylvania State College. This work is supervised by Otto Olson, who has been interested in investigations dealing with cigar-



This tobacco received 165 pounds of urea, 195 pounds of precipitated bone, and 600 pounds of sulphate of potash per acre, and was grown in a four-year rotation. It yielded 2,080 pounds per acre. (Ephrata Station)

leaf tobacco for a long period, and is thoroughly familiar with the agricultural conditions prevailing in that locality.

We believe that field experiments relating to the tobacco crop of this locality should conform, as closely as possible, to the general agricultural system now in operation and which, no doubt, was in vogue long before any tobacco investigations were conducted. This policy has been rigidly followed at the Ephrata Station, but the results of the investigations lead us to believe that some modifications could be made in the prevailing methods of growing tobacco for both yield and quality, without seriously interfering with the present general program.

The Lancaster county system of soil treatment is highly satisfactory as regards crop yields and the preservation of fertility, but the soil today is not necessarily as capable of producing as good a quality of tobacco as it was when first placed under cultivation. While we have no experimental data to show whether this is true or not, experience with virgin soils leads us to raise the question. While it is entirely possible for the farm soils of today to have as great a quantity of organic matter and the necessary mineral nutrients as virgin soils, attention must be given to differences which may exist between the character of the organic matter and the form of inorganic materials in virgin soils, as compared with the cultivated soils of today. In other words, it is imperative to consider the quantity and kind of organic matter, and the quantity and kind of commercial fertilizers added to the soil, as well as the time and method of application.

The Use of Stable Manure

One of the best forms of organic matter to add to soils is stable manure. The beneficial effects of a single application of manure have been known to persist for more than 50 years. Manure contains many beneficial micro-

organisms which multiply in the soil. In addition, it stimulates the growth and reproduction of those micro-organisms pre-existing in the soil, since it serves as a food material for these lower forms of plant life as well as those of a higher order. It must be remembered, however, that stable manure is, at best, an unbalanced fertilizer, with nitrogen the predominating element. Moreover, it stimulates those soil organisms which have to do with the fixation of atmospheric nitrogen, so that an application of manure may serve both as a direct and as an indirect source of soil nitrogen. This is a point that should be given consideration when questions pertaining to tobacco fertilizers are raised.

Nitrogen favors the vegetative phase of plant growth, hence excessive quantities delay the proper ripening of tobacco. Unripe tobacco cannot be utilized to advantage in the manufacture of high quality cigars. Moreover, if there is a considerable delay in ripening, there is likewise a danger from frost; frosted plants do not cure nor ferment properly.

The addition of stable manure immediately before the young plants are transplanted, invariably leads to a deficiency of nitrogen, since the available supply is appropriated for a time by soil micro-organisms. This condition may be largely controlled by the addition of a nitrate fertilizer to the manure before it is applied to the field, or shortly after. The use of stable manure, therefore, may lead to a lack of nitrogen during the early stages of plant growth, and to an excess during the latter stages.

Our experience leads us to believe that there is no valid reason for the use of stable manure alone as a fertilizer for tobacco. The yield is consistently less, the quality inferior, and the plants are less resistant to the invasion of disease, as compared with plants which draw their sustenance from a soil receiving applications of properly reinforced manure or other desirable soil treatments. Good quali-



Havana Seedleaf tobacco is carefully harvested at the Pennsylvania Lock Haven Station.

ty tobacco has been obtained through the use of commercial fertilizers alone. Hence we recommend that manure be applied to the soil elsewhere in the rotation rather than directly to the tobacco crop, so that its maximum beneficial effects may be obtained and its injurious effects may be reduced to a minimum.

Plowing under legumes is another method practiced by Lancaster county tobacco growers, in order to supply organic matter and nitrogen to the soil. This practice has a place under any progressive system of agriculture. A measurable quantity of soil constituents are absorbed and utilized during the growth of the legumes; these become available to succeeding crops, after decomposition has taken place. The practice of plowing under stable manure and legumes, which is so prevalent among tobacco growers, and the high nitrogen content of the tobacco crop, lead us to believe that the Lancaster county soils are relatively high in nitrogen.

What has been said does not necessarily minimize the importance of nitrogen in the growth of the tobacco plant. No plant food can compare with nitrogen in its desirable effects on growth and quality; this is known

to all who are concerned with this particular crop. No one would recommend nitrogen alone as a tobacco fertilizer; and no one would recommend a fertilizer lacking in nitrogen. A proper balancing of all nutrients is necessary.

The Use of Lime

A soil deficient in lime is not usually a very fertile soil. The general tendency is for a soil to lose considerable quantities of this material after being placed under cultivation. If the lime content is not maintained within certain limits, maximum crop production cannot be obtained. The rational use of lime, therefore, is to be recommended.

Lancaster soils are of limestone origin and the lime content appears to be retained to a considerable degree against the forces of leaching. Some of these soils can be cropped for a long period without a lime application being necessary. If a soil is able to produce a good crop of legumes, lime is not a limiting factor. A soil, however, can have too much lime, so far as the tobacco crop is concerned. We believe that this is a factor that is

not generally recognized by the tobacco growers.

The effect of a high concentration of lime in the soil is to render soil potash and potash applied in fertilizers more insoluble. Care should be taken, therefore, to avoid using lime where it is not necessary; and to avoid, as far as possible, those treatments, such as applying excess quantities of nitrogen-containing materials, which increase the availability of the lime in the soil.

In addition to rendering soil potash insoluble, our results show that a high concentration of lime tends to interfere with the direct absorption of this material by the plant, especially in a dry season. Under these conditions we find that the dry matter of the cured plants contains about 8 per cent of lime and about 2 per cent of potash. This is not desirable; the large amount of calcium is not helpful to the "burn," while the low content of potash is even more undesirable from the standpoint of burning quality. The plants also contain excessive quantities of nitrogen, which is harmful to the burning quality.

Rain appears to remove some of the

excess calcium and nitrogen from the soil without necessarily affecting the potash content. At the same time, there is a response in root development which aids in the increased absorption of potash. Our results show that under conditions of optimum rainfall, the plant contains less nitrogen and calcium, and more potash.

There is no doubt that some of the soils in Lancaster county need lime. On the other hand, we know of good tobacco soils that have received no lime for a period of 50 years and yet produce crops of good quality tobacco. Likewise, possibly there are some soils that are quite deficient in available nitrogen. In choosing a tobacco fertilizer formula, therefore, it is necessary to approximate the average soil conditions of the county. The recommendation of 1,000 to 1,200 pounds per acre of a 3-5-12 or a 5-5-12 fertilizer, depending upon the preceding crop and whether manure is used, is, we believe, fundamentally sound. This will increase the potash content of the tobacco, lack of which appears to be of first importance at this time.

VITAMIN STUDIES XVIII. THE BIOLOGICAL ASSAY OF FOOD MATERIALS FOR VITAMIN A AS INFLUENCED BY YEAST FROM VARIOUS SOURCES

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VITAMIN STUDIES XVIII. THE BIOLOGICAL
ASSAY OF FOOD MATERIALS FOR VITA-
MIN A AS INFLUENCED BY YEAST
FROM VARIOUS SOURCES*†

By

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IN making the biological assay of food materials for vitamin A we have followed, in principle, the curative procedure suggested by Sherman and Munsell (1) which requires a preliminary feeding (depletion) period to rid the tissues of vitamin A, after which sufficient vitamin A-carrying food is added barely to maintain body weight for a period of 8 weeks or to permit of slow growth not to exceed 3 grams per week. Some of our curative experiments, however, have been conducted for 5 instead of 8 weeks for the reason that we have felt that the shorter time was adequate for the information we desired to obtain. We have not found that the additional 3-week period has added much information and the shorter period is less expensive and time consuming.

In the past we have observed, from time to time, considerable variability in the growth response of rats during such experiments and Hume and Smith (2), Sherman and Burtis (3) and others have recorded similar observations.

The experiments described in this paper are the result of an attempt to point out that one of the reasons for divergent results in the assay of vitamin A may lie in the variability of the yeasts or other materials used as a source of the vitamin B complex. This question is not a new one, for Collison, Hume, Smedley-McLean and Smith (4), Hopkins (5) and Luce and Smedley-McLean (6) have already raised questions regarding the variability of yeast. The first-named (4) were of the opinion that their sample of marmite was deficient in vitamin G (B_2), while the others (5, 6) published evidence to show that certain preparations of yeast fat contained vitamin A. Most workers have assumed that yeasts do not contain vitamin A and Hume, Smith and Smedley-McLean (7) have shown that fats from certain

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† Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 504.

yeasts were devoid of this vitamin. Our results tend to show that yeasts may vary in their potency and that this may be due to the presence of vitamin A and to variability in the potency of the vitamin B complex.

In the experiments described in this paper, the rats were taken from our own breeding colony at 21 days of age, at which time they averaged about 40 grams in weight. Litter mates were distributed throughout the experimental groups to eliminate chances for error due to litter peculiarities and at least 10 animals were fed each type of experimental ration. Each growth curve, shown in the charts, represents the average of 10 or more individual growth records. The animals were fed separately in individual cages containing false bottoms made of wire mesh. The ration consisted (in parts per 100) of casein 18, agar 2, salt mixture (McCullum's 185) 4, (8) and dextrin 76. Yeast, in weighed amounts, was fed (daily) separate from the ration and distilled water, containing a trace of iodine, was furnished *ad libitum*. The antirachitic factor was furnished by irradiating the ration (9) with a quartz mercury vapor lamp.

In former years it has been our custom to feed 0.5 gram of yeast daily to furnish the vitamin B complex. Under these conditions we have been able to deplete the vitamin A reserves in 37 days, at which time the vitamin A-carrying food was introduced. The end of the depletion period is determined by a sudden and sharp drop in body weight or by a cessation of growth, or, occasionally, by a bloody nasal discharge or by the appearance of xerophthalmic symptoms. In all the experiments described in this paper, two drops of butter fat (66 mgm.) have been used as a constant and uniform source of vitamin A.

An examination of the records of 700 rats which had been used in various types of vitamin A experiments revealed the fact that not more than 20 per cent of our animals had shown evidence of ophthalmia. During this work we had used a uniform supply of dried yeast as the source of the vitamin B complex. Throughout this paper this yeast will be designated as Yeast I.

Yeast I was received at the laboratory late in 1925 and the supply was sufficient for the ensuing two years. It was with this yeast that we obtained an average growth of about 6 grams per week during the depletion period, which averaged 37 days in length.

In January, 1928, when the supply of Yeast I was nearly exhausted, a second shipment of yeast (Yeast II) was obtained from the same manufacturer. Additional feeding tests (with litter mates of some of the animals described above), identical in all respects except that Yeast II was substituted for Yeast I, gave disturbingly different results. Instead of 6-gram

gains in the depletion period we obtained an average gain of 10 grams for 5 weeks and the average length of the depletion period was extended from 37 to 42 days. The most significant difference, however, was the fact that 90 per cent of our animals were afflicted with definite ophthalmic symptoms as compared with 20 per cent in our previous work. We were confronted, therefore, with an anomalous situation. Our data indicated that Yeast I possessed antiophthalmic properties but poor growth-promoting properties, while Yeast II stimulated growth but possessed no power to prevent the development of the eye disease.

Although the supply of Yeast I was nearly exhausted, we were able to conduct a few experiments in which separate groups of animals received 0.5 gram daily of the two yeasts up to the end of the depletion period. At that point the yeasts were substituted one for the other and the experiment was continued. The ophthalmic symptoms in the group which had previously received Yeast II disappeared upon the introduction of Yeast I but the animals lost weight and died. These experiments led us to believe that we had some evidence to show that vitamin A may consist of two factors. Unfortunately, we were unable to obtain further data on this phase of the problem owing to our inability to obtain an additional supply of Yeast I. It seems evident that either vitamin A consists of two factors or that there is but one factor which has different and separate physiological rôles to play when fed at different levels. We are inclined to the belief that further work will substantiate the hypothesis that vitamin A consists of at least two factors, one which is specific in the prevention and cure of ophthalmia and other epithelial disturbances, and another which possesses the other physiological properties usually ascribed to vitamin A.

Yeast II was then extracted with ether and the ether extract and the yeast residue (now designated as Yeast II(x)) were fed, separately, to different groups of animals. When Yeast II(x) was fed as the source of the vitamin B complex the growth response was practically identical with that obtained with (unextracted) Yeast II except that the depletion period was shortened to 35 days. When the ether extract was freshly prepared and fed at the high level of 0.5 gram daily, growth was stimulated and improvement was observed in animals afflicted with ophthalmia, indicating that a trace of the antiophthalmic factor was present when the extract was fed at this high level, which was roughly 100 times the amount present in the daily allotment of unextracted yeast. At necropsy the animals compared favorably with the control groups which received two drops of butter fat daily. Post mortem examinations of all vitamin A-deficient animals which received Yeast I showed lesions in the lungs and occasionally in the ears.

Since we have been using Yeast III exclusively, the necropsies have revealed pus in the sinuses, both nasal and mastoid, in the throat, kidney, bladder and reproductive system, corroborating the findings of Tyson and Smith and others (10,11,12).

The vitamin A-free control groups died in 10 to 14 days. When this was repeated on Yeast III (produced by another manufacturer) the growth response was surprisingly good but the depletion period was shortened to 32 days, ophthalmia occurred to the extent of 100 per cent, and the animals died in 10 to 14 days following the depletion point, indicating that Yeast III was deficient in vitamin A. A study of the ether extract of Yeast III

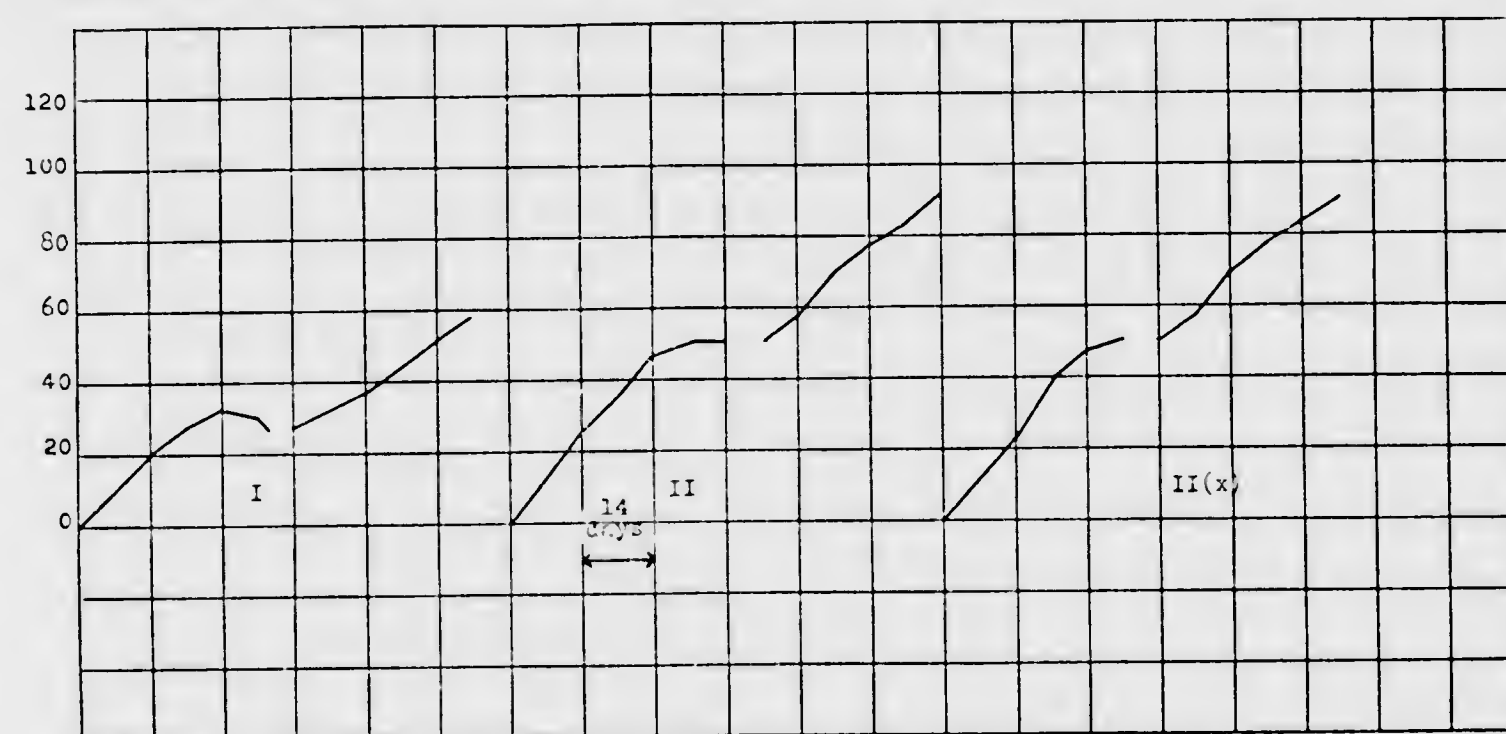


CHART 1.—Average growth curves of the groups which received Yeasts I, II and II(x), showing that the depletion period was lengthened when Yeast II was fed. All animals received 2 drops of butter fat daily during the curative period.

verified these conclusions. When butter fat was added at the end of the depletion period we obtained the greatest growth response we had ever obtained (see Chart 2). It appeared evident, therefore, that the potency of yeast from the standpoint of the vitamin B complex might be the cause of this pronounced growth response.

In order that we might obtain further light on the relation of the vitamin B complex to vitamin A response, four groups of rats were placed on the experimental ration. From the beginning to the end of the experiment each rat received 0.5 gram of yeast, the respective experimental groups being divided according to the type of yeast used. Two drops of butter fat were fed, daily, to each animal at the end of the depletion period. The only experimental variable was the type of yeast, the four groups receiving Yeasts I, II(x), III, and IV respectively. Yeast IV was a dried yeast which had been fortified with an addition of yeast autolysate.

An examination of Graph A, Chart 2, shows that growth response varied with the type of yeast employed. With Yeast I the growth rates during the depletion and curative periods were 6 grams and 6.4 grams, respectively; with Yeast II(x), 10 grams and 8.0 grams, respectively; with Yeast III, approximately 14 grams during both periods; while Yeast IV (from a third manufacturer) produced growth rates of 11 grams and 9 grams, respectively. These data have been corroborated in subsequent experiments. It

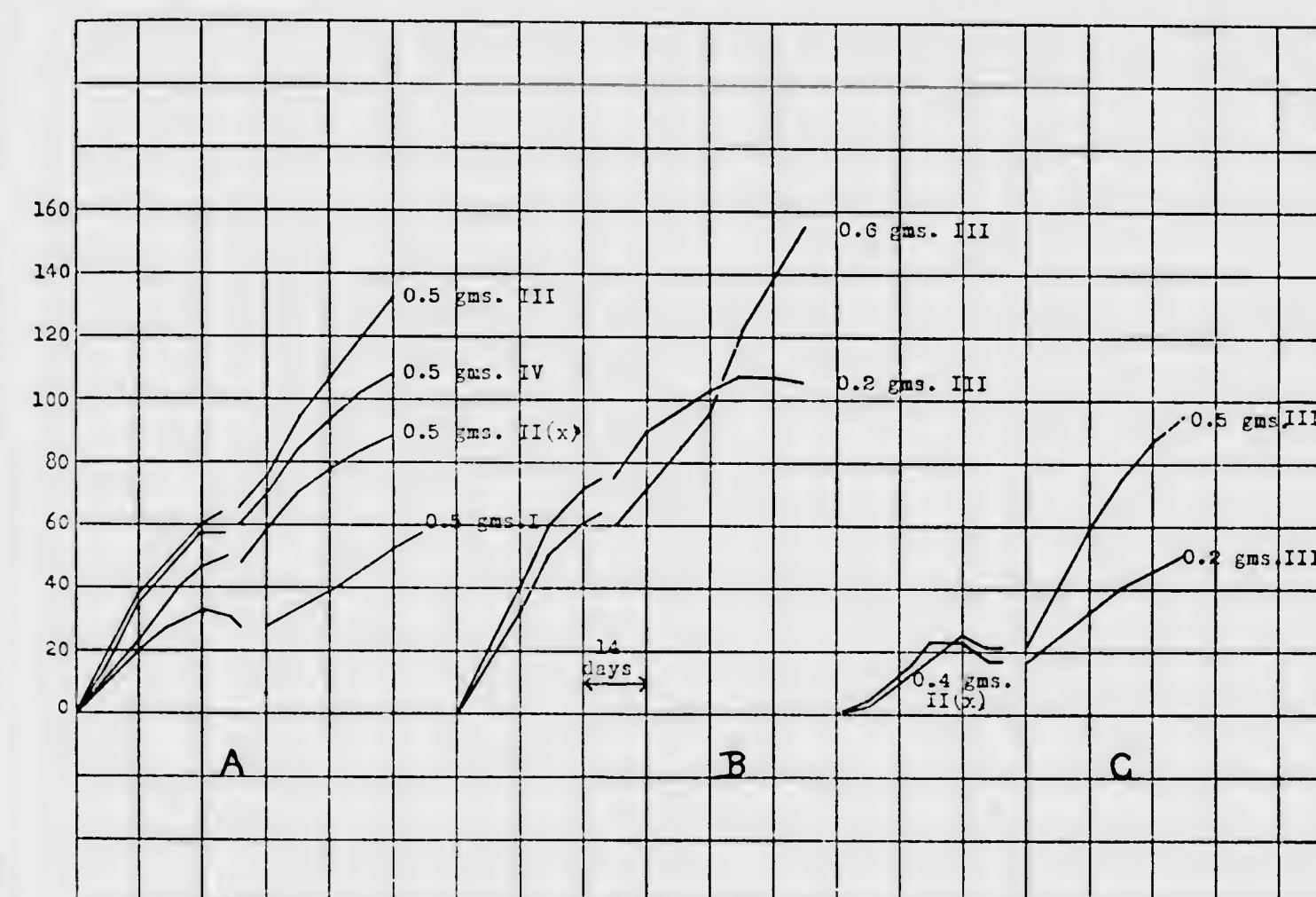


CHART 2.—Graph A shows the average growth curves of rats receiving 0.5 gm. of different yeasts (daily) throughout the experiment and 2 drops of butter fat daily during the curative period.

Graphs B and C show the effect of feeding yeast deficient in the vitamin B complex during the depletion period and changing to a better yeast at the beginning of the curative period. All animals received 2 drops of butter fat (daily) during the curative period.

seems evident, therefore, that the source of the vitamin B complex is a matter of greatest importance in the standardization of vitamin A technique.

Since different brands of yeast influenced the rate of growth when the source of vitamin A was kept constant, we tried the effect of substituting one yeast for another at the end of the depletion period. In Graph C, Chart 2, are shown typical growth curves obtained when two groups of rats received 0.4 gram of Yeast II(x) during the depletion period. At the end of this period both groups were about equal in body weight. At this

point Yeast II(x) was removed and 0.2 gram of Yeast III was substituted in one group while 0.5 gram of Yeast III was added to the diet of the second group. The feeding of two drops of butter fat was started simultaneously in both groups. It will be noted that the growth rate in the curative period was nearly doubled by the increased amount of Yeast III. Since we proved to our own satisfaction that Yeast III was devoid of vitamin A, we are forced to conclude that the increased response was due, in all probability, to the additional vitamin B complex added.

This experiment brings out two points worthy of emphasis: 1, that vitamin A response is correlated with the vitamin B complex supply in the ration, and 2, that the rate of growth after vitamin A administration is not necessarily a function of the weight of the animal at the beginning of the curative period. Sherman and Burtis (3) have pointed out that the weight of the animal at the beginning of the curative period is a most important factor, affecting subsequent response to vitamin A feeding. We agree with this observation but desire to emphasize that this is not necessarily the sole explanation.

Our assertion that body weight is not the sole criterion in this regard received additional support when another experiment was conducted where 0.6 gram of Yeast III was fed to two groups of rats during the depletion period. At the beginning of the curative period both groups received 2 drops of butter fat per animal but the amount of Yeast III was changed from 0.6 gram to 0.2 gram, while the other group was allowed to continue with 0.6 gram of Yeast III throughout the entire experiment. The results are shown in Graph B, Chart 2. In spite of the greater body weight at the beginning of the curative period, the group which received the reduced amount of vitamin B complex in the second period did not grow well after the amount of yeast had been reduced, the growth rate being 6 grams per week as compared with 19 grams per week for the group which received 0.6 gram of yeast throughout the experiment. This was the only experiment conducted in which the animals were all males, which accounts for the greater growth response. In all other experiments males and females were distributed uniformly among the groups.

In the final experiment 11 groups of animals were placed on experiment, of which 7 groups received Yeast III in graduated amounts ranging from 0.2 gram to 0.8 gram by 0.1 gram intervals. Four groups received Yeast II(x) in 0.2 gram intervals, the daily allotments being 0.2, 0.4, 0.6 and 0.8 gram, respectively.

An examination of Graphs A and B, Chart 3, shows that growth responses, in the depletion period and after the introduction of 2 drops of

butter fat per animal, vary with the amount of yeast fed. It is also instructive to note that 0.2 gram of Yeast III produced somewhat better growth than 0.8 gram of Yeast II(x). Here again we have evidence to show that the weight of the animal at the beginning of the curative period is not necessarily an index of the growth response to be expected upon the introduction of vitamin A, for the reason that those animals which received 0.2 gram of Yeast III grew better (11 grams per week) after the introduction

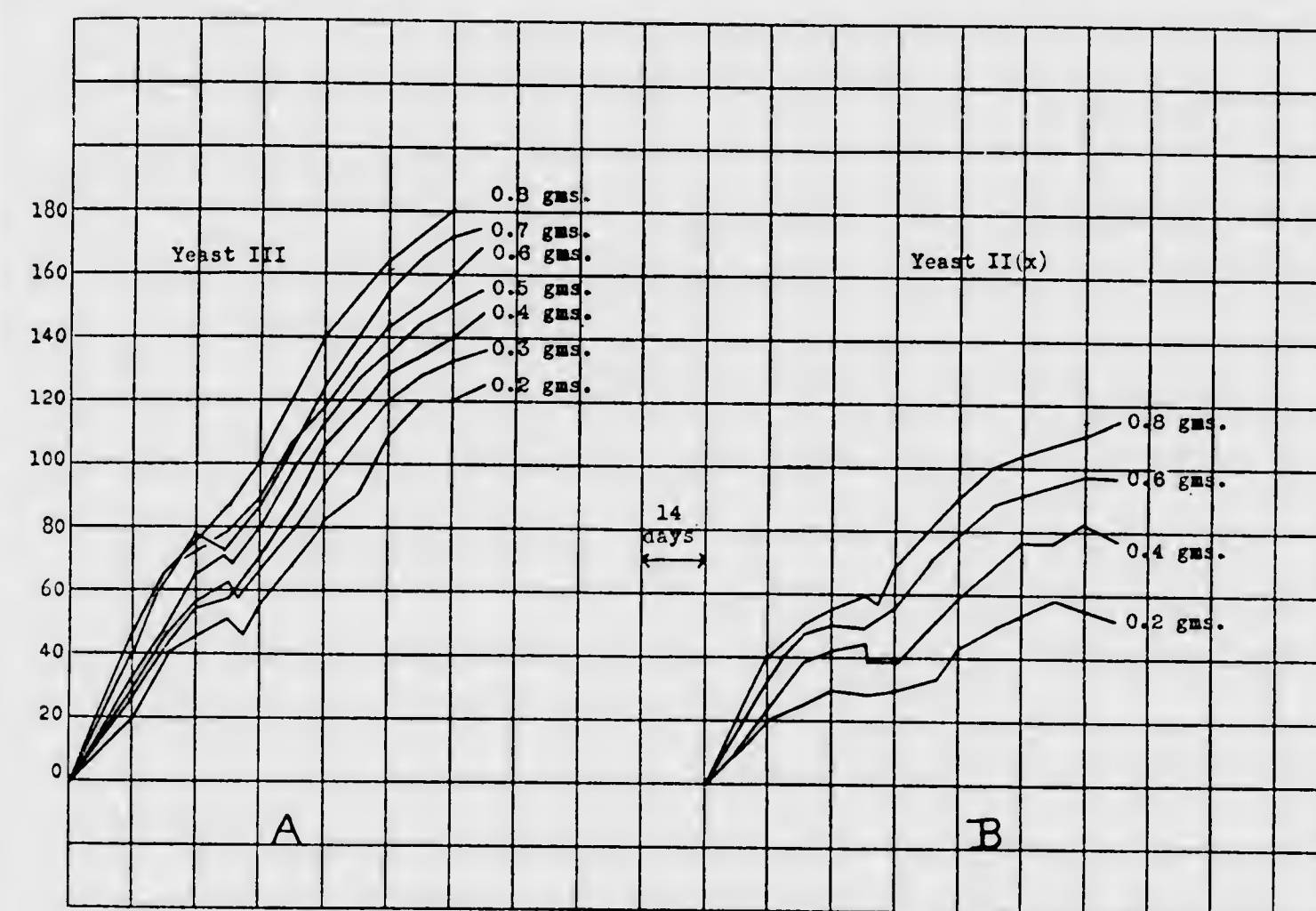


CHART 3.—Graphs A and B show the influence of graduated amounts of yeast on growth in the depletion and curative periods. All animals received 2 drops of butter fat (daily) during the curative period.

of butter fat than those animals which received 0.8 gram of Yeast II(x) and which averaged but 7.5 grams per week. At the beginning of the curative period, however, the latter were somewhat larger than the former.

Experiments are now under way with the hope of ascertaining the rôle played by the B (B₁) and G (B₂) fractions of the vitamin B complex in vitamin A response.

CONCLUSIONS

The results of this work lead us to conclude:—

1. That yeasts made by the same manufacturer may or may not contain traces of vitamin A.
2. That vitamin A response, using the curative method, will vary, depending upon the yeast used as the source of the vitamin B complex.

3. That quantitative results, in assaying foods for vitamin A, will vary, depending upon the potency of the vitamin B complex in the yeast employed.

4. That in the interest of accuracy, laboratory workers can well afford to make periodical tests of their new supplies of yeasts in order to prevent this variable from affecting their vitamin A technique.

5. That vitamin A may consist of two factors, one possessing antiophthalmic properties and which tends to preserve normality of other epithelial tissues, and the other factor possessing the growth-stimulating properties usually ascribed to vitamin A.

6. That our inability, in former years, to obtain ophthalmic symptoms in more than 20 per cent of our animals has been explained, due to the difference in antiophthalmic properties of yeast.

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5545

Effect of Irradiation on Electrokinetic Potential, Agglutinability, Lysis and pH of Escherichia Coli Suspensions.*

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(Introduced by R. Adams Dutcher.)

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From a study of the effect of irradiation of aqueous suspensions of *Escherichia coli* with the rays from a B carbon arc, the following conclusions, which harmonize with our hypothesis concerning electrokinetic potential as a measure of activity, stimulation, injury, recovery and death¹ have been drawn:

1. Storage and exposure to atmosphere of room in which the irradiation was carried out were not the cause of the change in electrophoretic velocity noted after irradiation, nor did they change the ability to be agglutinated.

2. Insertion of Corex A glass filter lessened the action of the ultraviolet radiation considerably. This suggests that the shorter wave lengths are more bactericidal and charge-reducing.

3. It has been shown [using both Northrop-Kunitz (maximum and actual values) and Falk capillary cells] that irradiation, if of sufficient duration, produces a decrease in negative charge which accompanies death.

4. Data obtained with the Northrop-Kunitz cell (both maximum and actual velocity) indicate an initial stimulative action of the ultraviolet radiation, which makes itself felt in an increase of negative charge. This increase of charge was not often observed with the Falk capillary cell.

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† The authors wish to acknowledge the invaluable assistance of G. W. Sharpless, K. P. Dozois, E. C. Holst, R. L. Ferguson, and Dr. W. P. Davey.

¹ Tittsler and Lisse, *J. Bact.*, 1928, 15, 105.

PROCEEDINGS

5. Short time irradiation, which merely stimulates or injures, permits of a return toward normal of electrokinetic velocities. Long time irradiation, which kills, produces a lasting effect.²

6. The time after irradiation at which comparative electrophoretic velocity readings are made must be carefully controlled if the irradiation is of brief duration.

7. Bacterial suspensions having practically the same charge can be prepared over a long period of time from different generations of the same organism.

8. Electrophoresis studies are more sensitive than the usual agglutination studies for detecting the effect of irradiation on the charge of *Esch. coli*.

9. The sequence of decreasing electrophoretic velocities is the same as that of increasing agglutinability except in the case of short (return to normal follows) and very long (lysis sets in) time irradiation.

10. The introduction of a Corex A filter practically causes this sequence of agglutinability to disappear.

11. Irradiation produced lysis which was greater the longer the irradiation.

12. Irradiation produced an increase in pH of the unrayed aqueous suspension whose pH was approximately 6.1. Since such change was also observed when the water itself was irradiated, it suggests the use of recently boiled water in future work.

13. Similar work, using 500 watt Mazda bulbs and Falk cells, showed eventual reduction of charge (which was preceded by a stimulatory increase), and evidence of lysis as indicated by clearing of the suspension. To accomplish similar effects, much longer times of irradiation were necessary than with ultraviolet rays.

14. X-rays produced no changes in electrophoretic velocities, nor did they produce bactericidal action.

Further evidence in favor of our hypothesis concerning electrokinetic potential as a measure of activity, stimulation, injury, recovery and death, is obtained from our work with the nitrogen fixing organism, *Rhizobium meliloti*. In general those cultures (history known) which were high nodule producers or high nitrogen-fixers had a higher negative potential than those of low abilities (Zucker³). An objection to the hypothesis is to be found in the work on heat killing of bacteria.⁴

² Osterhout, Injury, Recovery and Death. Lippincott, Phila., 1922.

³ Zucker, *J. Bact.*, 1929, **17**, 18.

⁴ Winslow, Falk, and Caulfield, *J. Gen. Physiol.*, 1923, **6**, 177.

IRRADIATION, POTENTIAL, AGGLUTINABILITY OF COLI

Since this work was begun, a number of statements suggesting these findings have appeared. Norton⁵ suggested that the changes that bacteria suffer when exposed to ultraviolet rays are accompanied, perhaps preceded, by changes in the electrical charges of the bacteria. Beaver and Muller⁶ stated that red gold sols change to blue on exposure to ultraviolet rays, but by prolonged irradiation they are peptized to stable red sols. Falk and Reed,⁷ working on the alterations in red blood cell electrophoretic potential produced by direct irradiation of blood *in vivo* reported a slight decrease of potential difference. Mayer⁸ says, "So far no attention has been paid to the importance of the nature of the electrical charge on a substance that is irradiated by ultraviolet light. A study of the effect of light on body tissues from this point of view promises interesting results."

A detailed account of these findings will appear in a technical bulletin of the Pennsylvania Agricultural Experiment Station. For progress reports see ^{9, 10}.

⁵ Norton, *Newer Knowledge of Bacteriology and Immunology*, Jordan and Falk. Univ. of Chicago Press, 1928, 374.

⁶ Beaver and Muller, *J. Am. Chem. Soc.*, 1928, **50**, 304.

⁷ Falk and Reed, *Am. J. Physiol.*, 1926, **75**, 616.

⁸ Mayer, *Clinical Application of Sunlight and Artificial Radiation*. Williams and Wilkins, Baltimore, 1926, 355.

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A COMPARISON OF COLORIMETRIC AND POTENTIOMETRIC METHODS FOR HYDROGEN ION DETERMINATION OF SOLID BACTERIAL MEDIA, USING A DILUTION METHOD BASED ON THE BUFFER EQUATION^{1,2}

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INTRODUCTION

In our unpublished studies concerning the effect of the medium on the electrophoretic velocity of the organism grown thereon, it became necessary to measure the pH of solid bacterial media. In the light of the errors exhibited by indicators, the question arose as to whether the colorimetric method (which is most commonly used in the bacteriological laboratory because it has the advantage of simplicity) is sufficiently accurate for this purpose. Whenever highly accurate values are necessary, the colorimetric values should be verified by potentiometric determinations. Previously, this has been done with liquid but not with solid media.

Since we were dealing with a solid medium the question arose as to how to make pH determinations on such a medium potentiometrically. It was thought that if the medium was sufficiently buffered, it would be possible to do this by diluting the melted agar until it remained a liquid at room temperature, an idea suggested by buffer principles.

¹ This is a résumé of a thesis submitted by O. G. Jensen in partial fulfillment of the requirements for the degree of Master of Science in Agricultural and Biological Chemistry in the Graduate School of The Pennsylvania State College, 1929.

² Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 513.

HISTORICAL

For the fact that the colorimetric method is not always accurate, the reader is referred to Sörenson and Palitzsch (1913), and Clark and Lubs (1917). Jaumain (1925) reports an error of 0.8 pH when using bromthymol blue to determine the pH of a 1/50 concentration of blood serum. On the basis of this he recommends that the indicator method be avoided for determinations of protein-containing materials. Schlegel and Stueber (1927) call attention to the acid error in the use of bromthymol blue. The salt error has been studied thoroughly by Kolthoff (1918, 1925). He found bromthymol blue among the indicators exhibiting the smallest error. Sharp and McInerney (1926) state that it is not advisable to use indicators at the limit of their ranges unless the determination is checked by the use of an indicator whose limits are well within the observed pH.

Cullen (1922) introduced a colorimetric method for determining the pH of blood serum and plasma. He determined a factor, by the use of which, one can convert the colorimetric pH value obtained at room temperature on a sample which has been diluted to 20 times its original volume, to the actual value at 38°C., as determined by the hydrogen electrode. Hastings and Sendroy (1924) report that the correction used by Cullen is not necessary when the readings are made at body temperature. They find that the average difference under these conditions is only 0.003 pH, the colorimetric value being the higher. Johnston (1928) and Myers and Muntwyler (1928) discuss this point at greater length.

The hydrogen electrode can be used where turbidity and color interfere with indicators. The bacteriologist does not use potentiometric methods to a large extent; they are too cumbersome, and it is seldom that reactions need to be determined more accurately than the colorimetric method permits. In the light of the above statements, however, he needs to be aware of the fact that colorimetric and potentiometric values do not always agree, and therefore, he must ascertain whether or not such a difference does or does not exist, and whether or not he is justified in using the simpler colorimetric method.

Up to the present time there is but one record of an attempt to measure directly the hydrogen ion concentration of a solid bacterial medium by means of a hydrogen electrode. Radsimowska (1924) claims that he was able to get an excellent agreement between the results obtained with his electrode and with the ordinary U-form electrode of Michaelis in the usual buffer solutions of Sörenson, Michaelis, and others. He states that a special report will contain the results of using this electrode to determine the pH of a single bacterial colony.

Biilmann and Lund (1923), and Biilmann (1927) have shown that the hydrogen electrode can be replaced by the quinhydrone electrode.

Biilmann and Krarup (1924) have determined potential differences at various temperatures with the quinhydrone electrode, and have given us an equation for calculating the potential of the electrode in respect to a hydrogen electrode at "t" degrees temperature. Recently, Biilmann and his co-workers (1928) tested the accuracy of the quinhydrone electrode in phosphate solutions, and found that the quinhydrone electrode gave correct pH values up to 7.73. Bayer (1926) found that with most soils constant readings were easily and quickly obtained, but that in case of alkaline soils the readings must be taken immediately after adding the quinhydrone. Lester (1924), working with dairy products found good agreement in values obtained by the hydrogen and quinhydrone electrodes. Watson (1927) has measured the pH of Swiss cheese by means of the quinhydrone electrode, but found a rapid drift especially in "green" cheese.

The change in potential of the quinhydrone electrode has been studied by several investigators and it has been generally conceded that the drift is due to a slow change in the ratio between the concentration of the quinone and the hydroquinone. Several factors may operate to bring this about. If the material contains oxidizing or reducing substances, the hydroquinone may be oxidized to quinone or the quinone reduced to hydroquinone. Bayer (1928) thinks that one of the substances may be selectively adsorbed when soil suspensions are used. Hissink and van der Spek (1927) are of the opinion that the drift is due to the fact that equilibrium has not been established.

Considerable variation in opinion exists as to the best time to make the readings. Hetterschij and Hudig (1927) came to the conclusion that readings taken immediately after adding the quinhydrone are the best. On the other hand Hissink states that thirty minutes should be allowed to elapse before readings are made. Corran and Lewis (1924) and Cullen and Earle (1928) find that good agreement is obtained between the quinhydrone and hydrogen electrodes when readings are extrapolated to zero time.

The following are the buffer equations that apply to solutions of weak monobasic acids in the presence of their salts and to weak monoacidic bases in the presence of their salts respectively:

$$[H^+] = K_a \frac{[\text{acid}]}{[\text{salt}]}, [OH^-] = K_b \frac{[\text{base}]}{[\text{salt}]}$$

The equations state that, as long as the assumptions upon which their development is based are valid (see Michaelis, 1926, p. 44), the pH of such solutions will not change on dilution for it depends solely on the ratios $\frac{[\text{acid}]}{[\text{salt}]}$ and $\frac{[\text{base}]}{[\text{salt}]}$, provided temperature is kept constant.

Several investigators have taken advantage of the fact that buffered solutions can be diluted without great alteration of the hydrogen ion concentration. Brown (1924) assumed that the pH of culture fluids does not change upon dilution. To determine the hydrogen ion concentration of small amounts of material, he added one drop to a small quantity of water and made a colorimetric comparison. Hastings and Sendroy (1924) and Hastings, Sendroy, and Robson (1925) showed that blood and urine can be diluted to great advantage. Cullen (1922) diluted blood plasma to 20 times its original volume with physiological salt solution. The turbidity of milk is a great hindrance to colorimetric determinations and for this reason Sharp and McInerney (1926) have studied the possibility of diluting the milk and thus removing the difficulty. In order to determine the dilution factor they plotted the pH of the original milk and of the milk diluted to various volumes against the logarithm of the volume

to which the milk was diluted. The curves obtained were invariably straight up to a volume dilution of 20 times. At this dilution the correction factor is -0.54 in order to bring the pH to the original value. This factor applies only to milk whose original pH is between 6.0 and 7.4. When the milk is as acid as pH 5.0 the factor diminishes to 0.22. Kolthoff (1928) states that the effect of dilution on the pH of buffer mixtures can be calculated.

THE PROBLEM

In order to determine whether or not colorimetric methods are suitable for the determination of the pH of solid bacterial media, it becomes necessary to determine pH potentiometrically. Instances have been cited to show that colorimetric methods may be in error and that it has been found advantageous to dilute blood, milk, and urine, and to apply a correction factor for the accompanying change in pH. In view of the nature of the constituents of nutrient agar, it seemed probable that its buffer capacity would be large enough to permit dilution to the point where it would remain liquid and the use of potentiometric methods would become feasible.

The hydrogen electrode is easily poisoned by protein-containing material, thus requiring frequent replatinizations. On the other hand, the quinhydrone electrode requires no platinizing and is simple to manipulate. For this reason, it was decided to determine the applicability of the quinhydrone electrode to these studies.

The problem consists of two parts: (1) A justification for the dilution; (2) a comparison of colorimetric and potentiometric methods, including both the hydrogen and quinhydrone electrode.

EXPERIMENTAL METHODS AND APPARATUS

The determinations were carried out on a medium made by dissolving 31 grams of dehydrated Bacto Nutrient Agar (Bacto-beef extract, 3 grams; Bacto-peptone, 5 grams; Bacto-agar, 15 grams, and sodium chloride 8 grams) and 1000 cc. of distilled water and autoclaving for twenty minutes at 15 pounds of pressure. Before the medium solidified, portions were diluted to 3, 5, 7, 10, and 15 times their original volumes, the distilled water

used having a pH of 6.0 ± 0.2 . Colorimetric determinations were made upon the undiluted medium and upon each of the diluted portions. Potentiometric determinations were made upon the portions that remained liquid. These were the portions diluted 5, 7, 10, and 15 times. In order to preclude any possibility of an error due to changes in pH with time, the determinations were made in alternating order.

Bromthymol blue, purchased from the LaMotte Chemical Products Company, was used for all of the colorimetric determinations. The set of comparison tubes was purchased especially for this study and kept in a refrigerator when not in use. The comparisons were made by the light from a Palo Daylight Lamp, thus avoiding the danger of dichromatism.

The apparatus for the potentiometric determinations centered about a Leeds and Northrup Type K Potentiometer, the null point instrument being an enclosed lamp galvanometer with a sensitivity of 0.025 microampere per millimeter scale reading. The known E.M.F. was furnished by an Eppley Cadmium Cell of the unsaturated Weston type. Its potential was 1.01878 volts at 20°C. No correction was made for temperature changes as the temperature coefficient is negligible between 15° and 35°C.

The hydrogen electrodes were of the Bailey type (1920). Two of the quinhydrone electrodes (nos. 2 and 3) consisted of platinum foils, approximately 4 x 8 mm. in size, while the other (no. 1) was made of 4 cm. of (24 gauge) platinum wire. A saturated calomel cell was the reference electrode. The hydrogen which was supplied from a cylinder was purified by passing it successively through solutions of alkaline pyrogallol, alkaline potassium permanganate, and saturated mercuric chloride. The quinhydrone was purchased from the Research Laboratory of the Eastman Kodak Company.

Each day, before any determinations were made, the standard cell was checked against a new cell of the same type which was kept as a standard. Following this the electrodes were filled with a standardized buffer solution and measurements taken to insure that the reference cell and each of the electrodes was in good condition. Any hydrogen electrode that was unduly slug-

gish or that gave readings more than 0.02 pH in error was discarded. The quinhydrone electrodes were cleaned in chromic acid cleaning solution and flamed with an alcohol lamp.

PRESENTATION OF DATA

It has been stated that many investigators have observed a drift in the potential of the quinhydrone electrode. We have found a similar change with nutrient agar. In order to determine the exact nature of the drift, we have thought it advisable to observe it over an extended period of time. Table 1 gives the

TABLE 1
Showing drift in the potential of the quinhydrone electrode

TIME*	E.M.F.	TIME*	E.M.F.
1	0.0261	20	0.0615
2	0.0297	23	0.0641
3	0.0328	25	0.0656
4	0.0359	30	0.0692
5	0.0384	35	0.0721
8	0.0449	40	0.0751
10	0.0488	45	0.0775
11	0.0504	50	0.0800
14	0.0547	55	0.0821
15	0.0560	60	0.0842
17	0.0583		

* Time in minutes.

results on a sample diluted to 10 times its original volume. Time is counted from the time of addition of the quinhydrone.

The data of table 1 are shown graphically in figure 1. It is evident that the curve over the interval from zero to five minutes does not deviate greatly from a straight line. For this reason the extrapolated values, tables 2 and 3, are made on the assumption that for the first five minutes the drift is practically a linear function of the time.

The pH values using the Bailey electrode were calculated as usual whereas those for the quinhydrone determinations were made by the method suggested by Biilmann and Krarup (1924),

except in one set of data which were calculated according to Cullen *et al.* (1925, 1928).

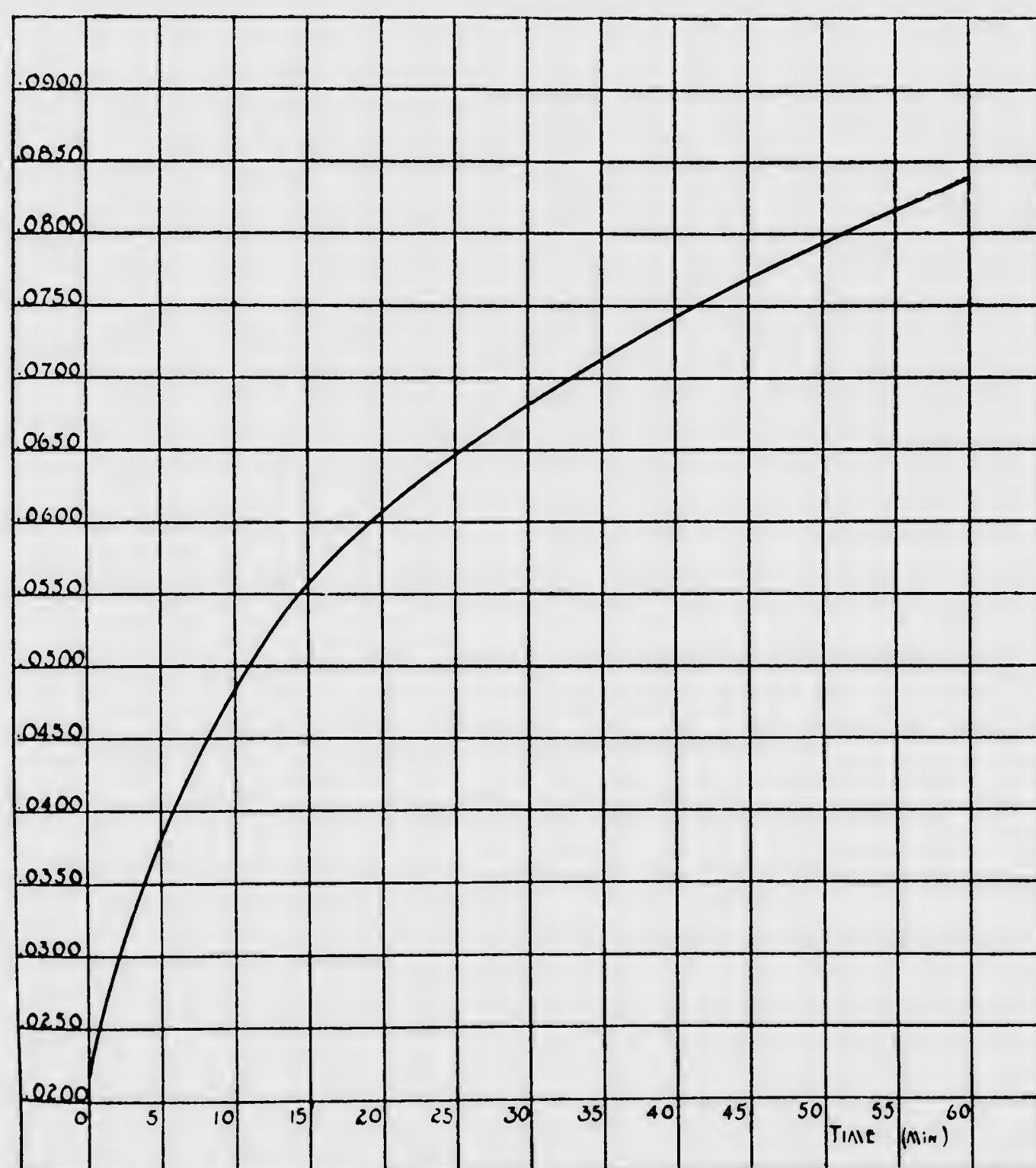


FIG. 1. THE DRIFT IN POTENTIAL OF THE QUINHYDRONE ELECTRODE

TABLE 2

A typical set of data obtained at the various dilutions by the three methods under consideration, at 25°C.

DILUTION	COLORIMETRIC pH	QUINHYDRONE ELECTRODE							HYDROGEN ELECTRODE—BAILEY		
		Electrode number	E.M.F. at 1 minute intervals after adding quinhydrone						Electrode number	E.M.F.	pH
			0	1	2	3	4	5			
None	7.2										
1:3	7.2										
1:5	7.2	1	0.0302	0.0324	0.0351	0.0372	0.0391	0.0411	1	0.6728	7.21
1:5		2	0.0293	0.0316	0.0333	0.0365	0.0396	0.0407	2	0.6731	7.22
1:5		3	0.0299	0.0325	0.0355	0.0384	0.0405	0.0430	3	0.6736	7.23
											7.22*
1:7	7.2	1	0.0355	0.0375	0.0387	0.0407	0.0422	0.0440	1	0.6717	7.20
1:7		2	0.0346	0.0366	0.0387	0.0403	0.0426	0.0444	2	0.6730	7.22
1:7		3	0.0355	0.0375	0.0397	0.0418	0.0427	0.0454	3	0.6731	7.22
											7.21
1:10	7.1	1	0.0377	0.0393	0.0410	0.0424	0.0442	0.0458	1	0.6714	7.19
1:10		2	0.0378	0.0394	0.0411	0.0427	0.0443	0.0456	2	0.6716	7.19
1:10		3	0.0385	0.0402	0.0417	0.0434	0.0445	0.0470	3	0.6720	7.20
											7.19
1:15	6.9	1	0.0503	0.0515	0.0533	0.0544	0.0550	0.0561	1	0.6614	7.02
1:15		2	0.0504	0.0516	0.0528	0.0541	0.0553	0.0562	2	0.6628	7.05
1:15		3	0.0515	0.0525		0.0546	0.0556	0.0565	3	0.6616	7.03
											7.03

* Average.

TABLE 3

Presenting average values from five sets of determinations made as per table 2

The average values are average hydrogen ion concentrations expressed in terms of pH.

METHOD	COLORIMETRIC						QUINHYDRONE ELECTRODE				HYDROGEN ELECTRODE—BAILEY			
	None	1:3	1:5	1:7	1:10	1:15	1:5	1:7	1:10	1:15	1:5	1:7	1:10	1:15
Dilution.....														
Set 1.....	7.2	7.2	7.2	7.2	7.1	6.9	7.15	7.06	7.02	6.80	7.22	7.21	7.19	7.03
Set 2.....	7.2	7.2	7.2	7.2	7.1	6.8	7.10	7.16	7.04	6.73	7.17	7.23	7.17	6.95
Set 3.....	7.2	7.2	7.2	7.2	7.0	6.9	7.01	7.03	6.93	6.84	7.16	7.17	7.15	7.04
Set 4.....	7.2	7.2	7.2	7.2	7.2	7.2	7.13	7.13	7.16	7.13	7.33	7.36	7.36	7.33
Set 5.....	7.2	7.2	7.2	7.2	7.2	7.2	7.23	7.20	7.18	7.14	7.28	7.31	7.31	7.31
Average.....	7.2	7.2	7.2	7.2	7.1	7.0	7.12	7.11	7.06	6.90	7.23	7.25	7.23	7.11

DISCUSSION OF RESULTS

It is quite evident that the original medium can be diluted to at least seven times its initial volume without a change in pH. From a knowledge of the buffer equation one would expect that dilution could not be continued indefinitely without a change in pH. Dilution of the agar to 10 and 15 times its initial volume caused a change in pH, as shown by all the methods used.

The drift in potential of the quinhydrone electrode was remarkably regular, successive readings giving values farther removed from the true hydrogen ion concentration. It is thought that the drift was due to a slow oxidation of the hydroquinone. However, even after excluding the effect of the drift by extrapolation to zero time, our values show that the quinhydrone electrode gives values which average 0.16 pH lower than those found with the hydrogen (Bailey) electrode. This intimates that there is another source of error not directly associated with the oxidation of the hydroquinone.

It was originally planned to use the bubbling electrode along with the Bailey type (1920) as a further means of comparison, but as consistent results could not be obtained, no data have been reported for it.

Clark and Lubs (1917) found that, for a liquid medium of about the same protein and salt content as the medium with which we worked, the colorimetric values were on the average 0.1 pH lower than the hydrogen electrode values. Our hydrogen electrode and colorimetric values agree well, showing that bromthymol blue does not exhibit either salt or protein errors in nutrient agar, and that the colorimetric method as ordinarily used gives accurate results.

SUMMARY AND CONCLUSIONS

A method, based on the buffer equation, has been devised for the determination of the pH of solid bacterial media, as it has been shown that the buffering power of nutrient agar is sufficient to permit a seven-fold dilution without a change in pH. At this dilution, the medium remains liquid, and the use of potentiometric methods is feasible.

Due to the cumbersomeness of the hydrogen electrode, the applicability of the quinhydrone electrode to pH determinations of nutrient agar has been determined. An effort has been made to eliminate the effect of the drift of the quinhydrone electrode by extrapolation of the values observed at successive intervals of time.

The average results of 60 parallel determinations indicate that the quinhydrone electrode gives values 0.16 pH lower than the hydrogen electrode.

It has been shown that bromthymol blue is an accurate indicator in nutrient agar, and that the colorimetric method agrees with the potentiometric method using the Bailey electrode.

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RANCIDITY¹

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Deterioration of fats through rancidity development has been a long recognized fact, but until recently there has been meager information available regarding the processes involved. This has been due in part, at least, to the conflicting views held regarding the nature and cause of rancidity.

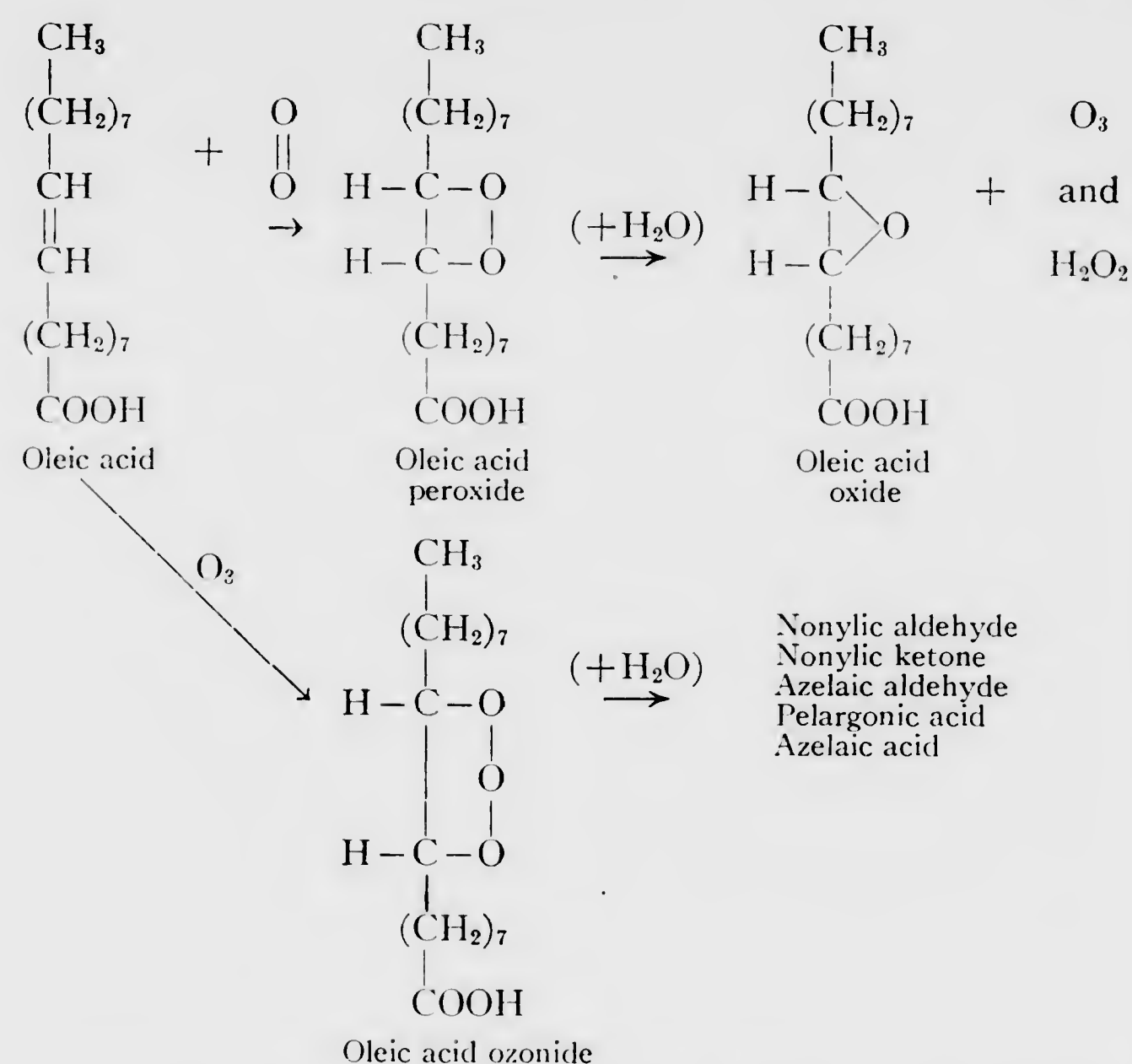
A survey of the literature will reveal three general types of fat deterioration, each considered as rancidity, although the processes of decomposition and end products obtained are very different. These three types of deterioration may be termed oxidative, hydrolytic, and ketonic rancidity. The development of oxidative rancidity is considered to be due to the addition of molecular oxygen to unsaturated glycerides with the formation of peroxide or oxide like compounds, which subsequently decompose into aldehydes, ketones, and fatty acids. This type of reaction may occur in all edible fats, since they all contain unsaturated glycerides, and is, for this reason, a very important type of fat spoilage. In the development of hydrolytic rancidity there is an hydrolysis of the glycerides with the liberation of free fatty acids as end products. This type of rancidity is of special importance in the spoilage of dairy products, due to the liberation of butyric acid with its characteristic odor and taste. The liberation of small amounts of the other fatty acids does not appreciably affect the odor and taste of a fat. Fats containing nitrogenous impurities, such as coconut oil, may undergo ketonic rancidity. This is effected through the action of certain molds on lower members of the saturated fatty acid series with the production of methyl ketones as end products.

Rancidity development in edible shortening agents and in baked goods is due ordinarily to oxidative deterioration. It is for this reason that this paper has been confined solely to oxidative rancidity.

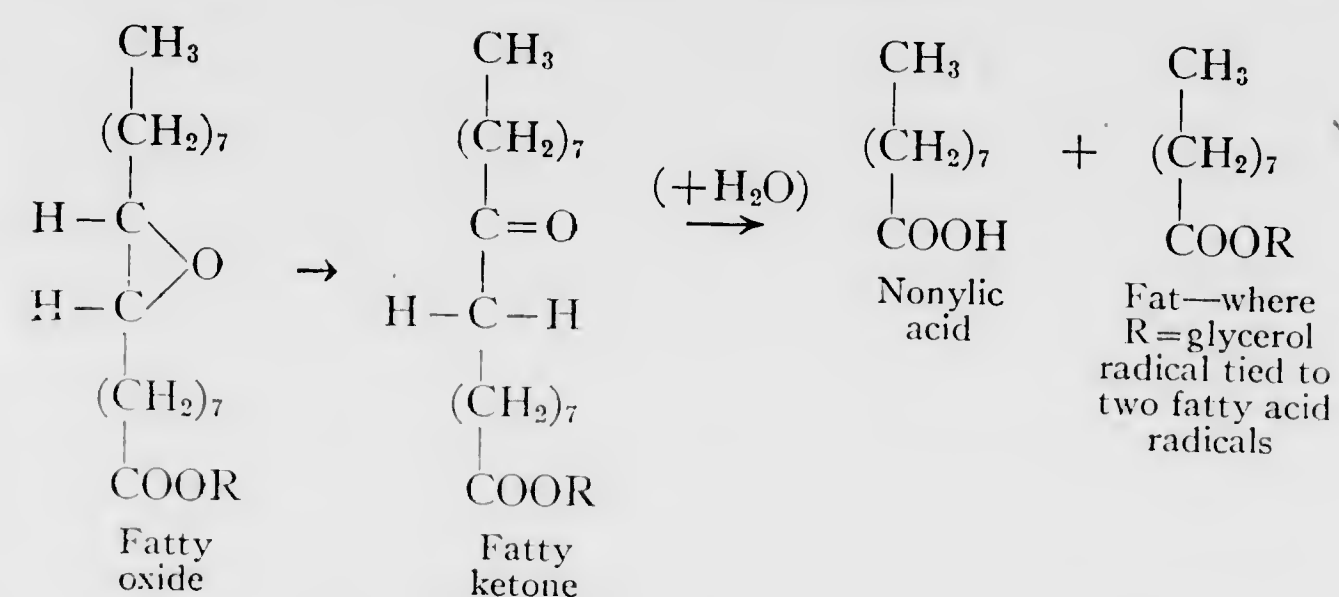
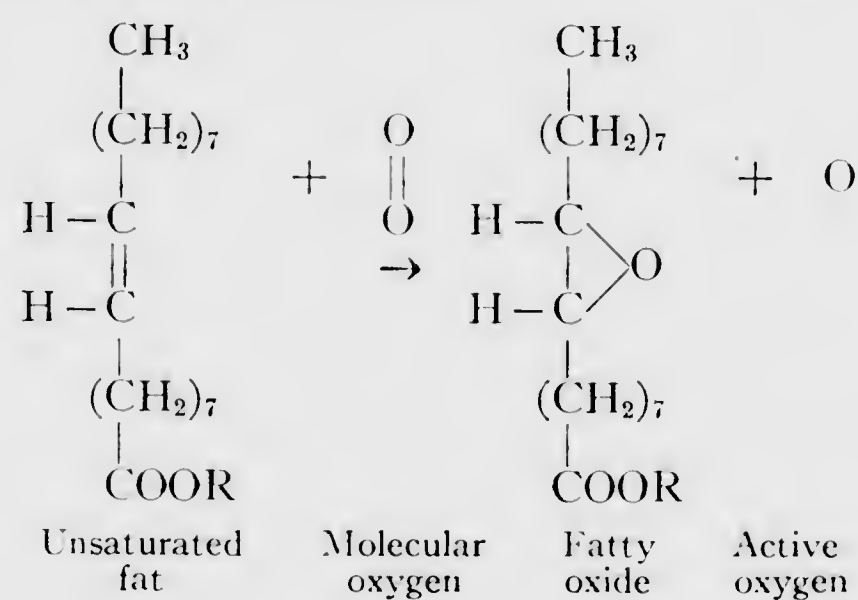
Theories Regarding the Development of Oxidative Rancidity

All the theories on the mechanism of oxidative rancidity have had their origin in the observation of Engler and Weissberg (1904), that molecular oxygen can attack "double bond" or "unsaturated" linkages, in a manner similar to that of ozone. The resulting compound of this autoxidation process contains a peroxide group and is

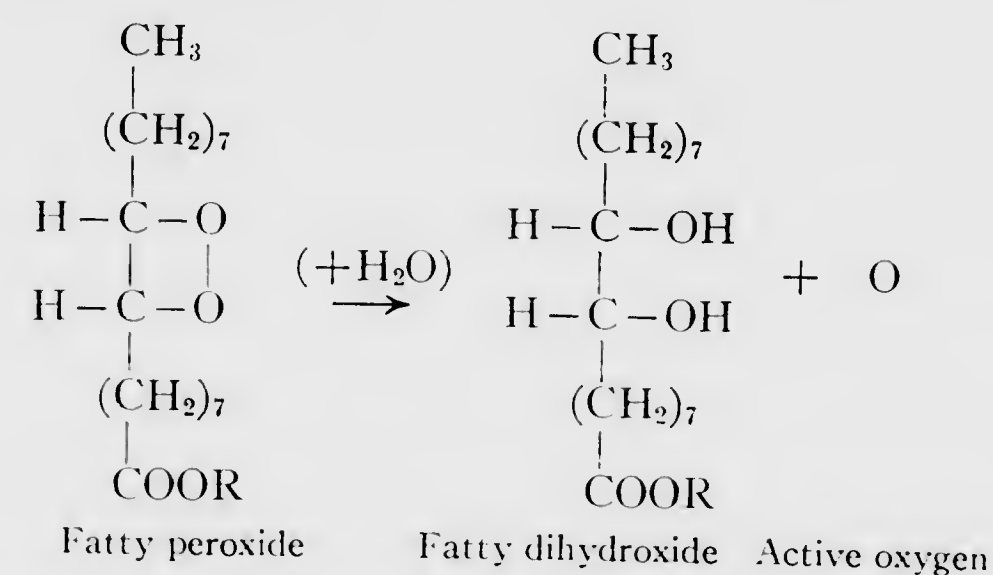
¹ Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as technical paper 529.



immediately acts upon the glycerides with which it is in contact and causes them to break up into free fatty acids, aldehydes, carbon dioxide, water and other decomposition products. Some of the active oxygen could also unite with the water present to form hydrogen peroxide. The fatty oxide, after rearranging to a ketone form might then decompose in the presence of water to a lower fatty acid. These reactions would proceed as indicated:



Browne also indicated in the event that a peroxide compound was formed, as postulated by Engler and Weissberg (1904) or Tscherich (1925), the reaction might go differently than these investigators stated. Such a peroxide might form a di-hydroxide with the liberation of active oxygen as indicated.



The various theories regarding the development of oxidative rancidity all agree that molecular oxygen attacks the unsaturated glycerides with the formation of a peroxide or oxide group at that point. These loosely combined oxygen compounds either decompose spontaneously into, or react with water to form aldehydes, ketones, fatty acids, active oxygen, ozone, and hydrogen peroxide. The liberation of active oxygen and ozone may cause the decomposition of glycerides, which would not ordinarily be attacked by molecular oxygen. No free glycerol can be detected in rancid fats, neither does the acidity of the fat need to increase with rancidity, consequently we assume that oxidation of the glycerides may take place independently of hydrolysis, a fact which is substantiated by the work of Nicolet and Liddle (1916).

Tests for Oxidative Rancidity

The first tests for rancidity were physical ones, depending upon the senses of taste and smell, and these are still the only tests upon which there is universal agreement by all investigators. Such tests, however, are apt to be influenced by personal opinion, and are hard to express in degree of rancidity. As a result, considerable research has been conducted to determine physical and chemical means for the detection of rancidity and for the numerical evaluation of its intensity.

The tests most commonly used have been color tests, of which the most important have been the peroxide, aldehyde, and Kreis tests. Peroxide tests were used by Vintesco and Popesco (1915), Kerr and Sorber (1923), and Powick (1923) for the detection of rancidity. These tests should be particularly applicable since peroxide compounds are the first products postulated in rancidity development. While the peroxide tests always react with a rancid fat, the intense color formed makes the evaluation of the degree of rancidity rather difficult.

Rancid fats also respond to the various aldehyde tests recommended by Schmid (1899) such as: (1) ammoniacal silver solution; (2) *m*-phenylene-diamine; and (3) 0.5% fuchsin sulphurous acid. Of these, only the fuchsin sulphurous acid test has been further recommended by Von Fellenberg (1924), who claimed that this test gave better results than the Kreis test. An extensive and complete comparison of the Kreis and Von Fellenberg tests was made by Pritzker and Jungkuz (1926) who concluded that the Von Fellenberg test had no advantage over the Kreis test. Just recently Schibsted (1931) has refined the test so that it is more sensitive and also permits an evaluation of the degree of rancidity. He expresses the content of aldehydes in a fat or oil in arbitrary color units obtained per unit of fat and suggests that this specific color value be called the "Fat Aldehyde Value." Schibsted found the improved reagent to be over 20 times as sensitive as any of the other reagents.

The most widely used test for the detection of oxidative rancidity is the Kreis test originated by Kreis (1902). The test consists in thoroughly mixing 1 cc. of a fat or oil with 1 cc. of concentrated HCl, and then adding 1 cc. of a 1% solution of phloroglucinol in ether. If after thorough mixing, the separated acid layer has acquired a red or pink coloration, the oil is rancid, and the intensity of the color is a rough indication of the degree of rancidity.

Kerr (1918) modified the test somewhat so that it served as a means of determining the degree of rancidity. He shook up 10 cc. of oil or fat with 10 cc. of concentrated HCl and 10 cc. of a 0.1%

solution of phloroglucinol in ether. If a red color developed in the acid layer, he diluted the original oil or fat with kerosene until a dilution was reached where no color reaction was obtained. The amount of dilution required was then an index of the degree of rancidity.

The main criticism of the Kreis test has been the inconsistent results obtained when the test has been applied to cottonseed oil. It was shown by Smith (1920) that naturally occurring substances were often present in cottonseed oil and would react to the Kreis test. These substances, however, were not glycerides and were not formed by the decomposition of glycerides, since the color produced in the reaction developed more slowly and was of a more purplish red shade than that produced by a rancid oil. Powick (1923) corroborated these results by a spectrophotometric study of the Kreis test, and demonstrated that by such an examination, it could be determined definitely whether the color was due to a rancid condition of the oil or to some other substance present.

In making a Kreis test, it is very essential to adhere to a rigid procedure, especially, if an evaluation of the color intensity is desired. If, for example, the order of adding the reagents is changed, colored solutions may be obtained of different intensities for the same amount of rancid fat and reagents. It is also necessary to insure that the reagents used are pure. Powick (1928) found that hydrochloric acid may contain nitrosyl chloride as an impurity, and as such, would give an intense red color with phloroglucinol but no color could be obtained when added to a non-rancid or rancid fat and phloroglucinol. Ether used in preparing the phloroglucinol solution or for extracting fat to be used in the test, frequently contains a peroxide contaminant which gives a reddish brown color in the Kreis test. Under such conditions, ether must be purified before it can be used. It is obviously necessary to always insure that a "blank" test, using only hydrochloric acid and phloroglucinol solution, gives no reddish color.

The intensity of the color produced in the Kreis test was found by Kerr (1918) to be roughly proportional to the degree of rancidity, while Holm and Greenbank (1923) found that the intensity of the color produced was directly proportional to the amount of oxygen absorbed by the fat. Since Kerr had based his results upon the dilution of a fat until it gave no color with the Kreis reagents, and Holm and Greenbank had matched the colored solutions obtained from the Kreis test with a set of standard color tubes, a somewhat more refined study of the test was undertaken with the aid of a spectrophotometer. It was desired to determine just how large a variation in intensity of the Kreis test might be expected between

samples of the same type of shortenings with equivalent oxygen absorptions. In the event that the difference between individual samples was not very great, such a study would be of considerable value in developing a method for determining the oxygen absorption (or degree of rancidity) of any sample of shortening.

The oxidation of the fat to definite oxygen absorptions was accomplished in a modification of the Holm and Greenbank (1923a) gas-tight stirring apparatus. The fat was oxidized at 95° C., samples being withdrawn at definite oxygen absorptions. Two grams of the oxidized fat were weighted into a small vial, and treated with 2 cc. of concentrated HCl and 2 cc. of a 1% solution of phloroglucinol in ether. After complete mixing, the contents were allowed to separate into layers, or centrifuged if necessary, to effect a separation. One cc. of the colored solution was then diluted to a 10 cc. volume with alcohol and filtered. A 2 cm. spectrophotometer tube was filled with this solution and examined in a Keuffel and Esser direct-reading spectrophotometer, and the percentages of light determined which were transmitted through the tube at the various wave lengths.

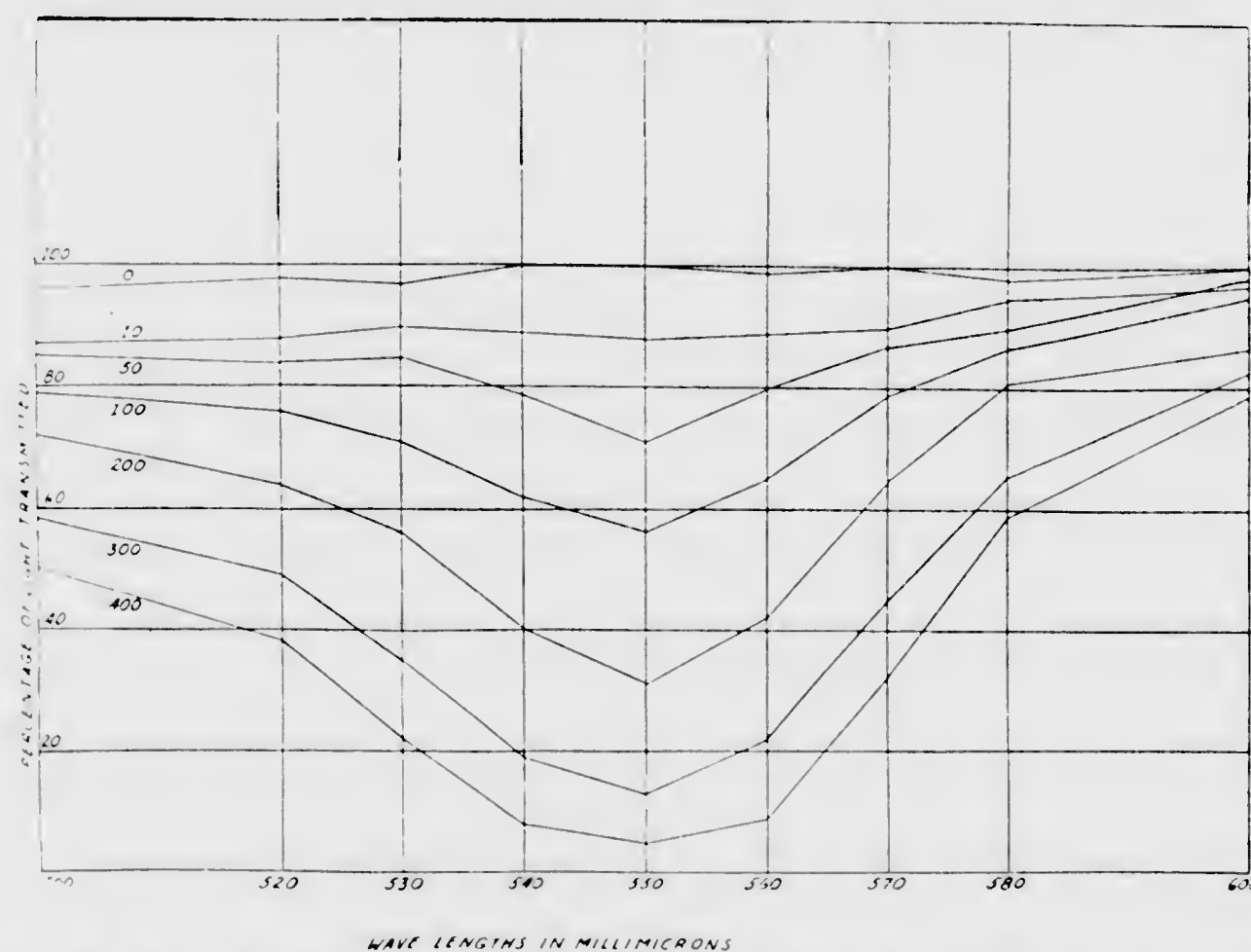


Fig. 1. Percentage of light transmitted at various wave lengths by the colored solution produced by the Kreis reagents on a prime steam lard with 0, 10, 50, 100, 200, 300, and 400 cc. of oxygen absorbed per 100 grams of lard.

It was found that the greatest absorption of light by the solutions occurred at the wave lengths 530, 540, 550, and 560 and 570 millimicrons. Figure 1 illustrates graphically the percentage of light transmitted at the various wave lengths by the colored solutions

obtained by the Kreis test on a prime steam lard sample with definite oxygen absorptions. Not only did the greatest absorption of light occur at these five forementioned wave lengths, but also, the percentages of light transmitted could be more easily and accurately determined at these wave lengths than at any others. For these reasons, it was deemed advisable to take the mean of these five readings as indicating the percentage of light transmitted by the colored solutions, representing a definite oxygen absorption by the fat. Since the percentage of light transmitted bears an inverse relationship to the percentage of light absorbed, or stated a little differently, to the color intensity of the solution, the reciprocal of the percentage of light transmitted indicates more clearly the relationship of oxygen absorption to the color intensity of the Kreis test on fats and oils. It was found that when the log of the reciprocal of the percentage of light transmitted was plotted against the amount of oxygen absorbed by the fat or oil, that practically a straight line relationship existed.

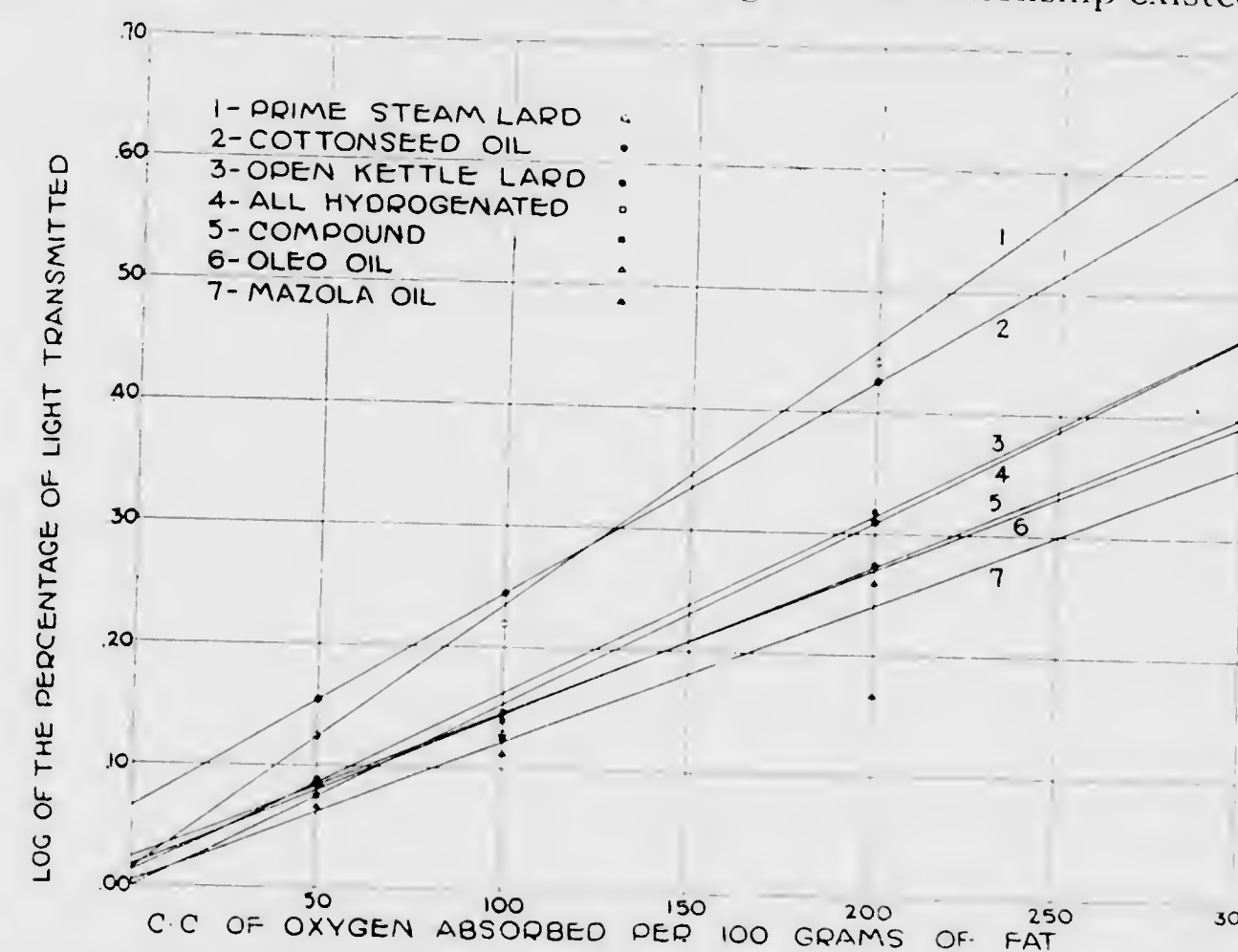


Fig. 2. Intensity of the Kreis test on various shortenings with definite oxygen absorptions. Intensity indicated by the log of the reciprocal of the percentage of light transmitted.

The most striking result indicated in the study (Figure 2) is that with the exception of cottonseed oil and prime steam lard, all shortenings with identical oxygen absorptions, gave Kreis tests of similar intensities. Cottonseed oil showed an initial absorption of light by the unoxidized oil, and if this is deducted, the results for the cottonseed oil approximate those for the all-hydrogenated shortenings. It would

appear from these results that the quantitative determination of the degree of oxidative rancidity could be fairly accurately determined for all shortenings especially at the lower oxygen absorptions by a spectrophotometric study of the intensity of the Kreis test. It should be emphasized that this work has only been of a preliminary nature and considerably more needs to be done before any definite conclusions can be drawn. These results were taken from some unpublished data obtained while at the University of Minnesota.

Methods for Studying the Susceptibility of Fats to Autoxidation

Various methods have been used to study the susceptibility of fats and oils to oxidative rancidity. Two of these methods, the controlled oxidation method of Greenbank and Holm (1925) and the methylene blue reduction method appear to yield the most promising results.

The controlled oxidation method consists briefly in weighing a definite amount of fat or oil into a flask, replacing the air within the flask by oxygen, and keeping the whole at a constant temperature in a carefully controlled thermostat. The time required for the sample to start reacting with oxygen, as registered by a fall in pressure within the system, is carefully noted and the amount of oxygen absorbed for definite intervals of time thereafter is determined.

The length of time a fat or oil is in intimate contact with oxygen before any consistent and measurable absorption takes place is taken as the induction period. The length of this period is then an indication of the ease with which a fat or oil will oxidize, or, is a measure of susceptibility to oxidative rancidity. This has been very clearly demonstrated by the results of Greenbank and Holm (1924) on butterfat.

The rate of methylene blue reduction in a fat under constant conditions has also been used as a means of determining susceptibility to oxidative rancidity. Greenbank and Holm (1930) have recently devised a method whereby the reduction of methylene blue by a fat is catalyzed by light and the point at which a definite reduction has taken place is indicated by a photoelectric cell. An appreciable decrease in the length of time of the experiment is obtained by this method.

A somewhat different procedure for performing the methylene blue test was used by Davies (1928). By this test, the fat to be examined is mixed with methylene blue and emulsified in skim-milk. This mixture is incubated at 37° C. and the blue color reduced by the milk reductase. After all the color has disappeared, the tubes are shaken vigorously for 15 seconds and allowed to stand 2 minutes.

The amount of restoration in color at the end of this period is a measure of the oxidation capacities of the fat or the ability to make use of the dissolved oxygen.

Factors Influencing Oxidative Rancidity

Controlled oxidation methods and methylene blue reduction tests have made possible the rapid and accurate determination of the effect of various factors upon oxidative rancidity. Thus, Greenbank and Holm (1924) found that fatty acids decreased the length of induction period of oxygen absorption of fats, the effect being greatest with the longer chain acids. Moisture, on the other hand, was found to increase the length of induction period of oxygen absorption, indicating a retarding effect upon oxidative rancidity. This is contrary to the role of moisture in hydrolytic rancidity, where it is essential for the hydrolysis of the glycerides. Fine and Olsen (1928) demonstrated the effect of moisture in slowing up the oxidative deterioration of stored grain products. I have obtained similar results with stored crackers, and so also have Holm, Wright and Greenbank (1927) in the case of powdered milks. Just how moisture retards rancidity development is not known. Greenbank and Holm (1924) believe that water may extract catalyzing substances from the fat or it may also cause the oxidation to go directly to the acid stage, instead of stopping at the aldehyde stage with the production of a rancid odor and taste.

Metals act as powerful catalysts in the oxidative deterioration of fats, only traces being necessary to effect a vigorous catalysis. This effect of metals is particularly a problem in the spoilage of dairy products, since traces of copper and tin are dissolved from containers in the various stages of processing of milk and milk products. Davies (1928) used the methylene blue reduction test to demonstrate the catalytic effect of metals in increasing the oxidation of butterfat.

High temperatures and light are also catalysts for the oxidation of fats, but of prime importance is the presence of oxygen or air. Storage in the absence of air, as in a vacuum, is a very efficient method of retarding oxidative deterioration in fats. Holm, Greenbank and Deysher (1927) have shown, however, that fat may contain loosely combined oxygen which can effect an oxidation of the fat even in a vacuum when stored over a considerable period of time. Displacement of the air by an inert gas such as nitrogen or hydrogen also prevents the rapid oxidation of fats, while carbon dioxide does not appear to act as an inert gas in this respect and does not materially retard the rate of fat oxidation. Emery and Henley (1922) found lard stored in an atmosphere of carbon dioxide became rancid as

quickly as when stored in air, while Supplee and Dow (1925) found the same to hold true in the storage of milk powder.

Anti-oxygenic Catalysts and the Autoxidation of Fats

Considerable interest has been aroused of late by the possibility of using anti-oxygenic catalysts to prevent or retard the autoxidation of fats. The study of anti-oxygenic catalysts was initiated largely through the results of Moureau and Dufraisse (1926), and has received its greatest impetus by the effective application in preventing the oxidative deterioration of rubber. The action of these substances as inhibitors has been explained by Moureau and Dufraisse on the basis of the theory of "negative catalysis" and by Alyea and Bäckstrom (1929) through the chain reaction theory. These inhibitors apparently function through forming compounds with, or being oxidized by, the peroxides in the fat, thereby decreasing the oxidizing potential of the fat and preventing its rapid oxidation.

In a recent paper Mattill (1931) has investigated the action of a large number of anti-oxidants on fats, and found a number of them (hydroquinone, quinone, pyrocatechol, pyrogallol, α -naphthol and B-naphthoquinone) effective as inhibitors. Mattill attempted to correlate the inhibiting power with the configuration of the inhibitor. His observations indicate that the anti-oxygenic effect is due, in the phenolic compounds, to two hydroxyl groups in the ortho or para positions, while in the naphthols but one hydroxyl group is sufficient.

The fact that substances are known which will retard the autoxidation of fats raises the possibility of their use in the preservation of edible fats. So far, at least, they have been viewed with suspicion due to their questionable toxic effect in foods, and their commercial application has not been attempted so far as we are aware.

It is interesting, however, that an anti-oxygenic substance appears to be normally present in wheat flour. Mattill (1927) found wheat germ oil to exert a protective action in preventing the oxidative destruction of vitamins A and E, and postulated that the sterols present in the oil were responsible for this action. This was later substantiated by Mattill and Crawford (1930).

Triebold (1929) studying the relation of shortening agents to the keeping quality of the crackers found certain cracker samples exhibiting much better keeping qualities than the shortening agents would warrant. He explained this on the basis that the wheat oil, present in the cracker flour, contained some substance which prevented the rapid oxidation of the shortening, an explanation, which is in direct contradiction to the more or less general idea that wheat oil aids in the oxidative deterioration of baked goods.

To test out the assumption that wheat oil acted as an inhibitor in the oxidative deterioration of baked goods, three series of cracker samples were prepared under constant conditions and their keeping qualities determined by the lengths of induction periods of oxygen absorption. One series of samples were baked as controls, another series were baked using the same flour as the controls except that it was first extracted with ether, and a third series used the flour after it had been simply treated with ether and evaporated without the loss of any of the oil.

The results obtained on these three series of cracker samples are given in Table I. It will be noted that the ether extracted flour,

TABLE I
EFFECT OF WHEAT OIL PRESENT IN CRACKED FLOUR ON THE KEEPING QUALITY OF CRACKERS

Sample	Length of induction period in hours. 100 gram samples of crackers and 20 gram samples of shortenings oxidized at 90° C.
Controls	7.0
Ether-treated (not extracted)	7.0
Ether-extracted	3.0
Prime steam lard shortening	4.5

from which the greater percentage of wheat oil had been extracted, showed a relatively short induction period, indicative of poor keeping qualities. Treatment with ether, without extracting the oil, did not affect the keeping qualities of the resulting crackers as they exhibited practically the same length of induction period as the controls. The baked crackers showed a considerably longer induction period than the original shortening, indicating that the keeping qualities of the shortening had been enhanced by the wheat oil in the cracker flour. This study has just been completed and we are continuing along this line at the present time.

Acknowledgment

The writer is indebted to Mr. Charles E. Bode for his aid in the extraction of the flour and the baking of the cracker samples.

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THE RECIPROCAL EFFECTS OF NITROGEN, PHOSPHORUS, AND POTASSIUM AS RELATED TO THE ABSORPTION OF THESE ELEMENTS BY PLANTS¹

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INTRODUCTION

Importance of understanding energy relations between soil and plant

Coincident with the rapid advances in physical chemistry, concepts with respect to soil fertility problems are undergoing profound changes. This is manifested by the results of recent investigations (1, 5, 8, 9, 25, 28, 83, 95) in which the principles of plant production are shown to be dependent not only upon a knowledge of the exchanges of energy in the soil, but also upon the energy relations of chemical reactions between the soil and the plant, including necessarily the transformations of energy taking place within the living cells. This paper will treat of the factors affecting the equilibria of nitrogen, phosphorus, and potassium in the soil in relation to the absorption of these elements by the plant.

A mathematical expression for evaluating the factors of fertility

It will simplify our presentation of the subject of the so-called "selective" absorption of nutrient elements from the soil if we can find an expression that will enable us to visualize the dominant soil factors affecting the nutrition of plants (35).

Influence of water.—Water is without question the greatest limiting factor in plant development. It is known from numerous experiments, especially from those of Hellriegel (19) and of Wollny (94), that for maximum growth there exists for each soil type an optimum water content. If Hellriegel's experimental results (19) on the variation of dry matter produced as a function of the water content of the soil are plotted, it is found that the points lie approximately on a parabola. Let A (fig. 1) represent the yield when the water content of the soil is optimum and let Y represent the actual yield. Since the curve obtained is a parabola,

$$\therefore k(x_i - x_o)^2 = A - Y \dots\dots\dots (A)$$

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i.e., the decrease in yield from the maximum is proportional to the deviation in the water content of the soil from the optimum, or

$$Y = A - k(x_i - x_o)^2 \dots \dots \dots (B)$$

where k is a constant expressing the sensitiveness of the plant to changes in the water content of the soil.

Water content of the soil in relation to the maximum efficiency of mineral nutrients.—But Wollny's experiments (93) show that the optimum water content of the soil corresponds also to the maximum efficiency of the mineral nutrients, i.e., to their maximum absorption. Since growth from balanced nutrient solutions is a function of the amount of the nutrient element absorbed, therefore, the absorption of a nutrient element is also proportional to

$$A - k(x_i - x_o)^2 \dots \dots \dots (C)$$

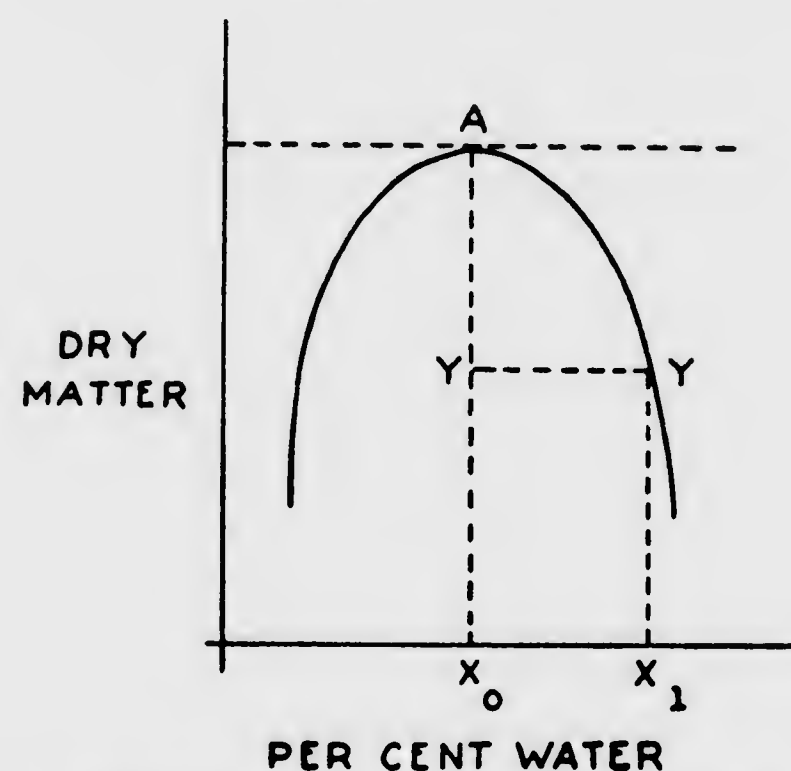


FIG. 1. RELATION OF YIELD TO WATER CONTENT OF SOIL

Let S_t represent the quantity of soil explored by the roots within a period of time t . Also let $\frac{1}{s}$ represent that fraction of the soil mass considered (S_t) entering into solution per unit of time in the form of a specific nutrient element (nitrogen, phosphorus, or potassium); and let $\frac{1}{r}$ be the fraction absorbed per unit of time by the plant. Then in a unit of time the fraction of the soil mass absorbed by the plant will be $\frac{S_t}{sr}$, where s measures the resistance to solution and r measures the physiological resistance of the roots to the absorption of the dissolved element (osmosis). The former can vary from $+\infty$ to 1. It is always great and depends on the nature of the soil. The latter can vary from $+\infty$ to 1 and depends on the feeding power of the plant (84, 89, 90, 91).

From (C) the absorption per unit of time is proportional to

$$A - k(x_i - x_o)^2 \dots \dots \dots (D)$$

Let us put

$$\frac{1}{sr} = K \left\{ A - k(x_i - x_o)^2 \right\} \cdot \frac{1}{s_i r_i} \dots \dots \dots (E)$$

The expression on the right represents then the fraction of the soil that passes from the solid condition to the plant in a unit of time.

Then, as we increase the time from t to $t + dt$, the amount absorbed (dy) will be

$$dy = K \left\{ A - k(x_i - x_o)^2 \right\} \cdot \frac{S_t}{s_i r_i} \cdot dt \dots \dots \dots (F)$$

where K is a constant representing the capacity of the plant. Or for the rate of absorption

$$\frac{dy}{dt} = K \left\{ A - k(x_i - x_o)^2 \right\} \cdot \frac{S_t}{s_i r_i} \dots \dots \dots (G)$$

In experimental work, of course, (dt) is relatively large; but it is remarkable that the average rate of absorption remains relatively constant for long periods of time (15 days, for example).

The equation shows that a soil can be deficient with respect to a given nutrient element for two reasons: (a) Inability, during periods of very active absorption, to maintain the supply above critical concentrations of the element. The function of fertilizers is to create a potential supply to provide for these emergencies. (b) Inability to provide at any time the supply of an essential element above critical concentrations. In this category are worn out or run down soils.

The equation modified by the addition of fertilizers.—Similarly for the rate of absorption of an element introduced in a soluble form such as a fertilizer which modifies a mass (say S') of the soil, it may be shown that the rate of absorption of the element introduced is expressed by the equation

$$\frac{dy}{dt} = K' \left\{ A' - k'(x_o - x_i)^2 \right\} \cdot \frac{S'_t}{s'_i r'_i} \dots \dots \dots (H)$$

The complete equation expressing the influence of the soil and of the added fertilizer is, therefore,

$$\frac{dy}{dt} = K \left\{ A - k(x_o - x_i)^2 \right\} \cdot \frac{S_t}{s_i r_i} + K' \left\{ A' - k'(x_o - x_i)^2 \right\} \cdot \frac{S'_t}{s'_i r'_i} \dots \dots \dots (I)$$

(fertility factor) (fertilization factor)

The formula clearly indicates the dominant rôle exercised by a soluble fertilizer in plant nutrition. As we shall see later the reciprocal influence of one element (ion) on another will involve the terms s and r of this equation.

The earliest experiments on the reciprocal effect of elements on absorption by plants

The reciprocal or mutual effect of one element upon another was first observed about the middle of the last century by Wolff (92). In his extensive nutrient solution culture investigations on the growth of barley, Wolff observed that the yield of plants grown in his "complete" nutrient solution was greater than that from cultures in which sodium was omitted. But Wolff's nutrient solutions were too high in potassium to be well balanced with respect to the concentration of this element. Analyses of the plants showed that the introduction of the sodium ion—from sodium chloride—decreased the absorption of potassium and so prevented its luxury consumption ("luxuskonsumption"), an expression which we have heard much about recently. Thus was it learned that absorption of an element in excess of that utilized might reduce yields and that this absorption in excess of the amount of the element required for the production of a normal plant could be controlled by the addition of another element—in Wolff's experiments, sodium. These experiments of Wolff's with sodium and potassium were confirmed by a number of investigators and definitely established the reciprocal action of at least two elements.

The concept of physiological balance

The existence of antagonism between other elements.—This reciprocal action of one element resulting in suppressing the absorption of another was later found to occur between other elements (ions) in the case of absorption by both plant and animal cells. A reciprocal relationship was found to exist between Ca^{++} and K^+ (10, 22, 27, 56), between NH_4^+ and K^+ (48, 49, 50), between Sr^{++} and K^+ (55), between Ca^{++} and Mg^{++} (51), between Na^+ and NH_4^+ (65, 66, 67), between SiO_2^{--} and PO_4^{--} (5, 16, 18, 43, 44), between Mg^{++} and Sr^{++} (55), between Mg^{++} and Ba^{++} (55), between Na^+ and Ca^{++} , between Na^+ and Mg^{++} (22, 27) and between K^+ and Fe^{+++} (15, 29).

Diminution of antagonistic action with dilution.—Loeb (49) gave the term "antagonism" to this phenomenon. But Osterhout (68) showed later that the antagonistic action increased with the increase in the concentration of the solution. In very dilute solutions, such as that of the order of magnitude obtained from soils by the various displacement methods, no antagonistic effect was observed. It follows that if the same mechanism is operative in soils as in nutrient culture solutions, it must be postulated that plant roots obtain their nutrients from a much more concentrated solution than that obtained thus far by artificial means.

Optimum ratios.—These investigations by Loeb and Osterhout led to the development of the concept of physiologically balanced solutions, from which followed the long search by means of nutrient culture solutions to discover the

best (optimum) ratios between the various nutritive elements (7, 20, 32, 47, 52, 53, 54, 73, 74, 76, 77, 78, 79, 88).

Nutrient culture versus field experiments

Absorption from soil and nutrient solutions compared.—The principles acquired by means of these laboratory studies cannot be overestimated with respect to the service rendered in providing a system of physiological knowledge for application to field studies. The environmental conditions, however, affecting the absorption of elements from the soil are different from those under which nutrient culture experiments are conducted because: (a) The rate of diffusion of ions toward the root zones is more rapid in nutrient culture solutions than in the soil (21); (b) the extent of the root system and, therefore, the absorbing surface is greater under field conditions than in nutrient culture solutions. The factor $\frac{1}{s_i r_i}$ of equation (I) thus becomes unity in solution but not in soil cultures.

Soil versus plant analysis.—Because the concentration of the soil solution with respect to any of the principal elements is subject to wide fluctuations even in the uncropped condition (2, 3, 80), it is difficult to draw conclusions relative to absorption of ions from soil solution studies alone. It is possible, however, as we shall see by comparing the amounts of nutrients absorbed—at the different vegetative periods—by plants growing under uniform soil and meteorological conditions, to extend very considerably our knowledge of the factors influencing the absorption of the principal (dominant) elements (ions) from soils.

THE LAW OF THE MINIMUM AND ITS IMPLICATION

Many "laws" have been advanced purposing to define the relationship between the amount of nutrients absorbed by the plant and the dry matter produced (45, 46, 60). The first was by Liebig (45), who announced as a corollary to what afterwards became known as the "law of the minimum" that nutrient elements must be absorbed in certain definite proportions and in such a manner that when the rate of supply of one of the principal nutrient elements is reduced below the critical concentration for that element the rate of absorption of the other principal nutrient elements is retarded or depressed, because an inadequate supply will render the other elements unavailable, since the plant will be unable to grow to any extent. Consequently, assuming all the other growth factors constant, it follows from Liebig's basic hypothesis that if, with time as the abscissa and the quantity of nutrient element absorbed during the growth of a plant as ordinate, graphs are plotted indicating the course of absorption of nitrogen, phosphorus, and potassium from a soil to which a "complete" fertilizer has been added—with all other growth factors constant—the graph showing the course of absorption of any one of these elements from a fertilizer containing only two of them would be displaced from its former position and would now fall below it.

The results of field experiments

The American field experiments.—The results which we have obtained in our experiments on the absorption of these elements by apple trees grown in the field under controlled conditions (86) are shown graphically in figure 2. The data are expressed in percentage of the element absorbed by the last season's branch growth on a moisture-free basis. These results are in accordance with Liebig's basic hypothesis. Thus, it will be observed that the omission of any one of the elements—nitrogen, phosphorus, or potassium—from the fertilizer has resulted in a decreased absorption of the remaining elements and is accompanied by decreased growth and reproduction. Analytical data recently

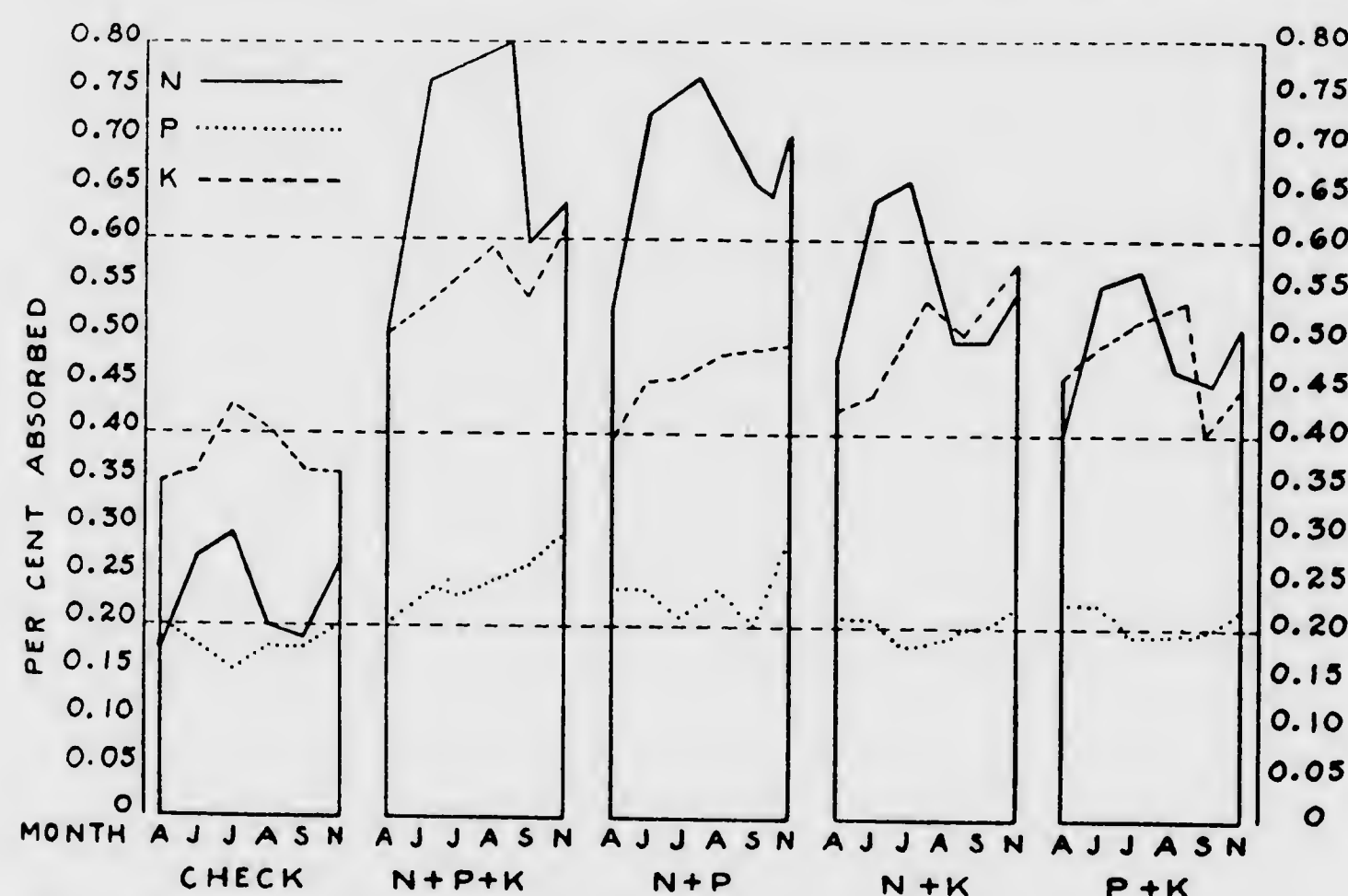


FIG. 2. THE COURSE OF THE ABSORPTION OF NITROGEN, PHOSPHORUS, AND POTASSIUM BY *Pyrus malus* L. AS DETERMINED BY THE PERIODIC ANALYSIS OF THE CURRENT AND SEASON'S BRANCH GROWTH

Results are expressed in percentages of dry weight of material

obtained with respect to the total amount of each element taken up by an entire tree show the same relationships.

The French field experiments.—But the results of very carefully conducted long time experiments by Lagatu and Maume (34, 36, 37, 38, 40) in Montpellier on the course of absorption of nitrogen, phosphorus, and potassium by the vine (*Vitis vinifera*) show that in wet as well as dry years the omission of one of the three elements—nitrogen, phosphorus, potassium—from the fertilizer resulted not in a decreased absorption of the remaining elements relative to that from the complete fertilizer, as would be expected from Liebig's law, but on the contrary in decreased yields produced by a nutritional lack of balance caused

by an increase in the absorption of the remaining elements of the fertilizer. The results shown in figure 3 are given in percentages of the dry weights of leaves.

These results are of paramount importance in the science of plant and animal nutrition and in their application to the problem of the fertilizer requirements of soils. They show the impossibility of making a comparison between the composition of plants of the same species unless the data are referred to the same standard control, i.e., to the composition of the plant of the same species grown under such

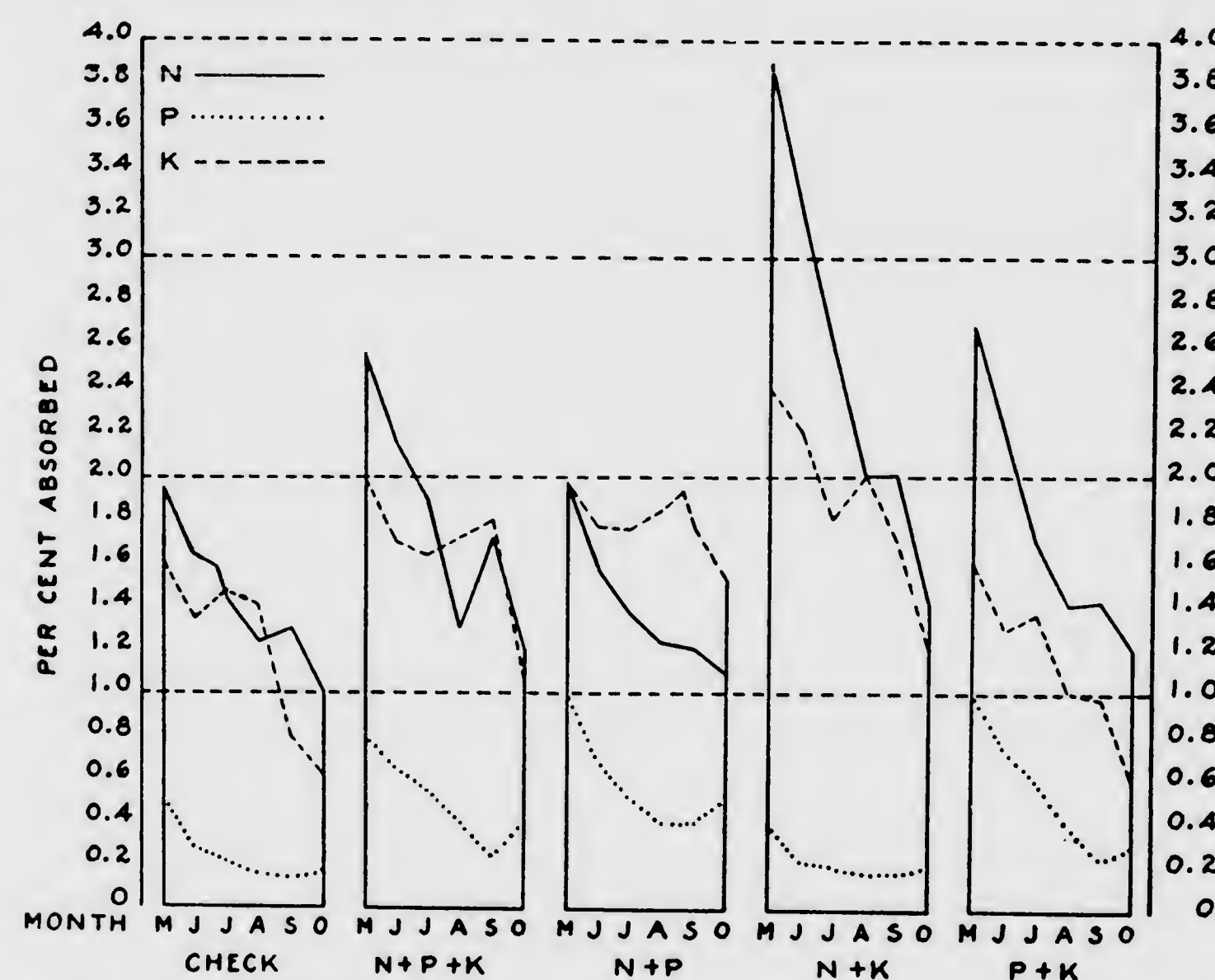


FIG. 3. THE COURSE OF THE ABSORPTION OF NITROGEN, PHOSPHORUS, AND POTASSIUM BY *Vitis vinifera* AS DETERMINED BY THE PERIODIC ANALYSIS OF THE LEAVES

Results are expressed in percentages of dry weight of material

conditions of physiological balance as to produce sufficient growth to ensure reproduction of the highest order.

It is important to ascertain the causes of these apparently anomalous results. But before attempting an explanation the results of some experiments in Austria on barley (75) bearing on this same problem will be given.

The Austrian field experiments.—Both nitrogen and phosphorus in the soil of this experiment are very low but the potassium content is high. The results, which are given in absolute amounts of the element absorbed (fig. 4), show that relative to the absorption of elements from the (N + P + K) plot the

omission of potassium resulted in an increased absorption of phosphorus and nitrogen and that relative to the (2N + P + K) plot the reduction in nitrogen (N + P + K) resulted in a decreased absorption of potassium and phosphorus.

In the unfertilized plot potassium, as would be expected from Liebig's law, is absorbed in relatively greater amounts than nitrogen or phosphorus up to the time of blossoming. The analyses of his plants, however, showed that after this period potassium migrated again to the soil. Sekera maintains that this migration is a physiological necessity and represents the plant's attempt to rid itself of an element absorbed in excess of that utilized.

It will be observed that when nitrogen and phosphorus are added (N + P) the absorption of the elements is increased and potassium does not migrate back to the soil, for the excess of potassium is now balanced by the increased absorption of nitrogen and phosphorus. The addition of potassium to this soil already abundant in this element, as will be seen from the (N + P + K)

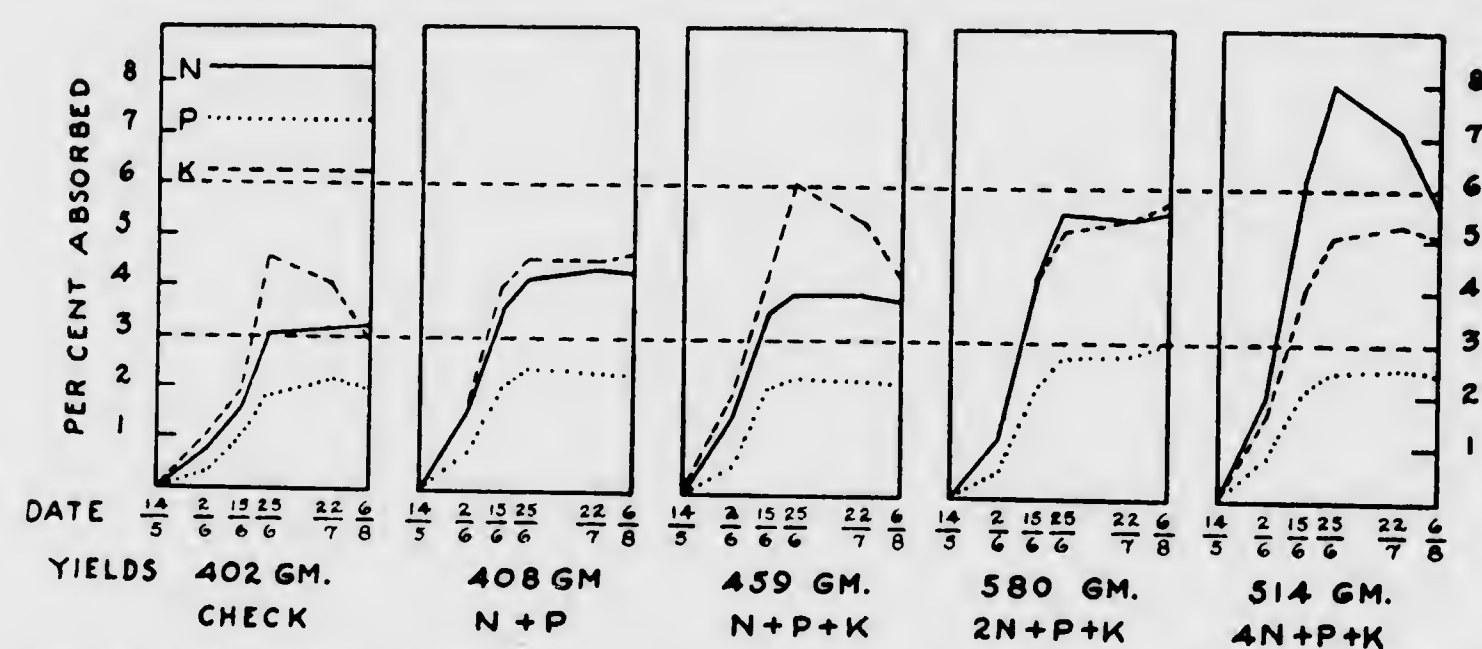


FIG. 4. THE COURSE OF THE ABSORPTION OF NITROGEN, PHOSPHORUS, AND POTASSIUM BY *Hordeum sativum* AS DETERMINED BY THE PERIODIC ANALYSIS OF WHOLE PLANTS
Results are expressed in percentages of dry weight of material

plot, causes a migration of potassium to the soil and a decrease in the amount of nitrogen and phosphorus absorbed. On the further addition of nitrogen, the absorption (2N + P + K plot) again becomes balanced and results in a maximum yield. Further additions of nitrogen result in an excess absorption of this element with migration to the soil and a decreased absorption of potassium and no influence on the absorption of phosphorus.

An analysis and synthesis of the results

Lagatu and Maume (35, 39, 40, 41, 42) have announced that their results clearly show that the corollary from Liebig's "law of the minimum" is not capable of generalization. The present writer will show, however, that the contradiction is an apparent and not a real one.

It may be seen from equation (I) that the effects of the addition of nitrogen,

phosphorus, or potassium to a soil, added singly or in one or more combinations, will—all other factors remaining constant—depend upon the factor $\left(\frac{1}{s_i} + \frac{1}{s_j}\right)$, i.e., (a) upon the supplying power of the soil to maintain concentrations of an element above the critical concentration, and (b) to the concentration with respect to that element produced by the added fertilizer. A knowledge of this supplying power with respect to a given nutrient element would, therefore, appear to be essential in order to determine the ratio between the constituents of a fertilizer necessary to provide a balanced soil solution. Unfortunately, the process of obtaining a true measure of the supplying power with respect to a given nutrient ion is laborious, involving long and expensive experiments (80).

For the purpose of the present analyses, however, sufficient information may be obtained from laboratory methods similar to those of König (33), and of

TABLE 1
Amounts of total solutes and of "available" nitrogen, phosphoric acid, and potash in the soils from the three experiments considered

LOCATION OF EXPERIMENT	DRY SOIL			
	Total solutes	Soluble in 1 per cent K_2SO_4	Soluble in 1 per cent citric acid	
		N	P_2O_5	K_2O
	p.p.m.	p.p.m.	p.p.m.	p.p.m.
State College, Penna, U. S. A.	310-490	82	65	130
Montpellier, France.	2,000-2700	260	1,300	210
Vienna, Austria.	(low)	47	280

Neubauer (61, 62, 63, 64, 87) and to the displacement (31, 69, 72) or extraction methods (2, 3). Information of this type is available (39, 40, 41, 42, 75, 85) for the check plots of the three experiments discussed. This information is assembled in table 1.

It will be observed that when the soil is deficient in an element, i.e., when s_i of equation (I) is high (or when $\frac{1}{s_i}$ is small), the concentration being below the critical level for that element (e.g., nitrogen and phosphorus in the Pennsylvania and Austrian experiments), the omission of the deficient element from the fertilizer has resulted in a decreased absorption by the plant of the remaining elements contained in the fertilizer added. Under these conditions the omission of the element from the fertilizer must result in a greater relative deficiency of this element compared with the other two elements and, accordingly, limits (decreases) their absorption as predicated by Liebig's "law."

But when an element is relatively abundant, i.e., when s_i of equation (I) is small, or when $\frac{1}{s_i}$ is large [e.g., nitrogen and phosphorus in the French experi-

ments (34, 36, 37, 38, 39, 40, 41, 42) and potassium in the Austrian experiments (75)], the omission of that element from the fertilizer would not reduce $\frac{1}{s_i}$ of equation (I) below the critical concentration for that element. Consequently, the omission of an element already abundant in the soil from a fertilizer results in an *increased* absorption by the plant of the remaining elements added in the fertilizer *relative* to the absorption of these elements from the complete fertilizer plot until the element omitted from the fertilizer becomes a limiting factor, i.e., until its concentration is reduced below the limiting concentration. *The whole problem, therefore, is one of relativity.* In no case is Liebig's law violated.

It should now be apparent that all statements in the literature bearing on the seemingly contradictory results of one worker with those of another on the problem of the mutual action of one element on the solubility of another and of its absorption by plants can be satisfactorily explained in the light of the foregoing interpretation.

The so-called "luxury" consumption of elements

All the experimental evidence (75) appears to indicate that the plant, especially in the early stages of growth up to blossoming, absorbs the nutrient ions in approximately the same proportion that these ions are present in the nutrient medium. Accordingly, if $\frac{1}{s_i}$ or $\left(\frac{1}{s_i} + \frac{1}{s_i'}\right)$ of equation (I) is so great as to be out of physiological balance for any element, a "luxusconsumption" (93) will occur. And, as we have seen, such "luxury consumption" may result in a disturbance to normal metabolism sufficient to affect growth and reproduction (34, 36, 37, 38, 39, 40, 41).

Sekera's experiments (4, 75) which give clear evidence that the excess of an element above that which the plant utilizes in metabolism may be returned to the soil shortly after blossoming occurs have already been discussed. Ions, therefore, may be expelled from the plant in the process of adjustment to more favorable equilibrium conditions. Hoagland's experiments (22, 27) would lead to the view that the mechanism of this *expulsion may be one of exchange of anions or of cations.*

Sekera's experiments (75), moreover, show that within the limits set by the amount of water and solar energy available the return of an element absorbed in excess of that required may be prevented by balancing it through the addition of the other two principal (dominant) nutrient elements (ions), thus enabling the plant to utilize this excess (fig. 4). These experiments, moreover, are of additional interest in indicating a method by means of which the required physiological balance between nitrogen, phosphorus, and potassium may be obtained.

No "luxury" consumption indicated in the old fertility plots of the Pennsylvania State College.—It is of interest to note that results obtained on the absorption

of nitrogen, phosphorus, and potassium by wheat in the old fertility plots having the highest yields of the Pennsylvania State College (56) do not indicate any absorption of an element in excess of that utilized. A comparison of the amounts of these elements absorbed by wheat on plots 17, 19, and 21, receiving increasing amounts of nitrogen as dried blood, shows that the highest application of nitrogen (72 pounds) has not resulted in a lack of balance. The results are given in table 2. We may deduce from this that yields might be further increased by increasing the rate of application of one or more of the carriers of the nutritive elements used in these plots.

TABLE 2
Amounts in pounds per acre applied and recovered in entire wheat crops, of nitrogen, potash, and phosphoric acid on dry basis (after MacIntire)

PLAT NUMBER	TREATMENT	NITROGEN		POTASH		PHOSPHORIC ACID	
		Applied to soil	Removed by plant	Applied to soil	Removed by plant	Applied to soil	Removed by plant
16	6 tons manure	59	34.8	52	37.6	38	18.5
17	(1N)PK	24	29.3	100	42.0	48	17.6
18	8 tons manure	78	39.2	69	36.5	51	19.8
19	(2N)PK	48	37.7	100	44.6	48	20.0
20	10 tons manure	98	44.4	86	46.0	64	22.2
21	(3N)PK	72	45.3	100	61.7	48	24.2
22	6 tons manure + CaO	..	36.4	...	34.9	..	21.6
24	Check	..	18.4	...	16.1	..	9.3

Definition of an optimum fertilizer ratio

Physiological balance within the plant once obtained may again be easily disturbed by the addition of any one of the principal nutrient elements to the soil. *The optimum ratio of the constituents of a fertilizer may then be defined as that ratio which, on the addition of any one of its nutrient constituents—nitrogen, phosphorus, or potassium—results in no increased utilization by the plant of any of the other elements as determined by the time-absorption-graph method.*

The extension of the concept of ionic balance to all elements

The influence of calcium ions.—For the purpose of simplification, the effect of the reciprocal action of elements on absorption has been confined in the present discussion to the three principal nutrients—nitrogen, phosphorus, and potassium—because under the conditions commonly encountered these three elements are the dominant and, indeed, controlling regulatory factors in plant metabolism (71), the functions of which cannot be replaced by any other element. Nevertheless, in practice it is necessary to take cognizance of the effect of other elements and especially of calcium when applications of lime are made regularly. The effect of calcium oxide (or carbonate) on the solubility

of soil nitrogen, phosphorus, and potassium will depend upon the physical and chemical-state in which they are present. On the old fertility plots of the Pennsylvania State College CaO and CaCO₃ have resulted in depressing the solubility of soil potassium (2, 3). Moreover, this depression in solubility is associated, as would be anticipated, with a decreased *absorption* of potassium by the plant (56).

THE MECHANISM OF THE RECIPROCAL ACTION OF IONS IN THEIR RELATION TO ABSORPTION

The nature of the functioning mechanism has not as yet been elucidated. The amount of an ion absorbed in unit time is expressed by the fraction $\frac{1}{sr}$ of equation (E), i.e., by the product of the solubility and osmotic value of the respective ions. It is dependent, therefore, upon the structure, chemical composition, and pore space of the root-absorbing surfaces on the one hand, and upon the degree of hydration, atomic volume, and the magnitude of the dissociation constant of the ions, on the other (17, 24, 57, 58, 59, 81, 82).

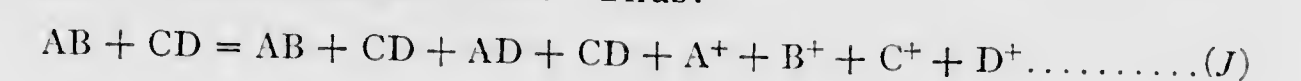
The relation of dissociation constants to antagonism

The significance of this latter influence is indicated by the experiments of Maume (57, 58, 59) on ion antagonism. In the case of binary electrolytes antagonism is related to the conductivity of the salts after mixture. The antagonistic effect is greatest at that particular concentration of ions represented by the intersection of the dissociation curves of each salt separately determined at dilutions corresponding to the proportions at which each salt is present in the mixture and which is indicated on the abscissa SS' (fig. 5). Thus, in the case of NaCl and CaCl₂, of all possible proportions of 0.12 Mol. NaCl (soln. S) and 0.164 Mol. CaCl₂ (soln. S₁) the least toxic mixture is at the point A of intersection of the dissociation curves represented by a mixture of 5 cc. CaCl₂ + 95 cc. NaCl.

Antagonism is positive.—In the case of binary mixtures of alkali and alkaline earth salts having a common anion the maximum antagonism occurs when the quantity of each salt is such that if separately dissolved in the same volume of water as that of the mixture this would give the same coefficient of ionization. This is indicated by the point represented by the intersection of the curves in figure 5. In this case the common anions will be at different concentrations. For example the molar concentration for NaCl, CaCl₂, Ca(NO₃)₂, and NaNO₃ having the same conductivity are 0.12, 0.22, 0.086, and 0.029, respectively.

Antagonism is nil.—The antagonism is *nil* when, as in the case of the chlorides of the monovalent ions Na⁺, K⁺ and NH₄⁺, for example, the dissociation constant of the mixture is approximately unaltered. This holds when salts having a common ion have, at equal molar concentrations, the same value for the dissociation constant.

Antagonism is negative.—The antagonism is *negative* when, as the result of double decomposition, the degree of dissociation is increased by the formation of different kinds of molecules and ions. Thus:



Causal factors as yet undetermined

These experiments of Maume are of interest in pointing the way to an explanation of the mechanism of antagonism between ions of the *same* electrostatic charge. Nevertheless, because of the present transition stage of our

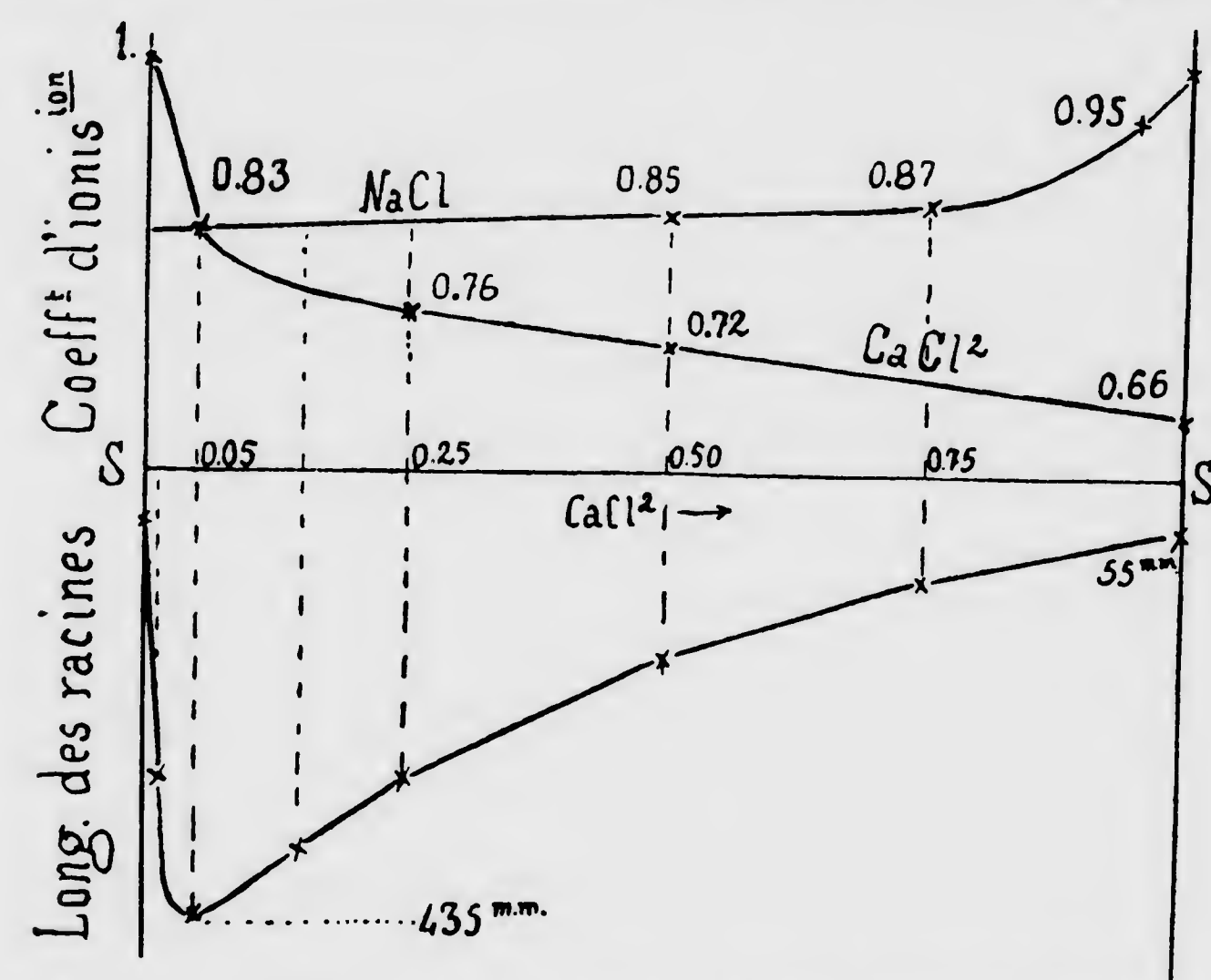


FIG. 5. RELATION OF THE LENGTH OF THE ROOTS OF WHEAT SEEDLINGS GROWING IN SOLUTIONS OF 0.12 MOL. NaCl (SOLUTION S) AND OF 0.164 MOL. CaCl₂ (SOLUTION S₁) IN THE PROPORTIONS INDICATED TO THE DISSOCIATION CURVES OF THE SEPARATE SALTS DETERMINED AT DILUTIONS CORRESPONDING TO THE PROPORTIONS AT WHICH EACH SALT IS PRESENT IN THE MIXTURE

knowledge concerning the dissociation of strong electrolytes, it would appear that the dissociation constant is only a measure of a causal factor that is as yet obscure. The influence of the motility of ions in relation to dissociation must be considered in determining the actual causal factors operative.

THE INFLUENCE OF OTHER FACTORS IN PRODUCING SELECTIVE ABSORPTION BY PLANTS

The absorption of the so-called physiologically acid and alkali salts

The relation of hydrogen-ion concentration to the absorption of the principal nutrient elements is gradually being elucidated by the application of the rapidly

accumulating facts of physical chemistry. By such means it is possible not only to explain but also to predict the physiological effects of applications of salts such as sodium nitrate and ammonium sulfate.

The rate of absorption of ions under the conditions of hydrogen-ion concentration existent in normal soils (pH 5.5 — 7.0) follows the lyotropic series of Hofmeister (30), viz., for cations the series, in descending order of mobility, is $H > K > Na > Li > Mg > Ba > Sr > Ca$ and for anions $OH > \frac{Bi}{I} > NO_3 > Cl > HPO_4 > SO_4$ (17). But at hydrogen-ion concentrations outside the aforementioned range the series is irregular and non-predictable (17).

The effect of the hydrogen-ion concentration of the substrate.—A reciprocal action resulting in "selective" absorption may take place between a cation and an anion not only in the presence of a non-diffusible ion (11, 12, 13, 14) but also when no non-diffusible ions are present. If the substrate is slightly acid, neutral, or alkaline, the membrane (roots) will, as a result of the absorption of OH^- ions, become negatively charged. The electrostatic attraction resulting from this potential difference will result in a more rapid absorption (osmosis) of cations than of anions (28). Under these conditions the cation is dominant. But, on the other hand, in an acid medium (especially pronounced below certain critical values *circa* pH 5.5), the membrane (roots), as a result of greater absorption of H^+ ions, becomes positively charged and the electrostatic attraction resulting from this potential difference causes anions to be absorbed faster than cations (28). Under these conditions the anion is dominant. The results of the investigations of Hoagland (23, 26), of Butkevitch (5, 6) and of Hager and Stollenwerk (17) on the absorption of ions from solutions of $NaNO_3$, $Ca(NO_3)_2$, NH_4OH , $(NH_4)_2SO_4$, and NH_4NO_3 may be interpreted in this manner. *The relative effects, therefore, of these salts may be altered at will by changing the concentration of the hydrogen-ion.* The process may be visualized as follows:

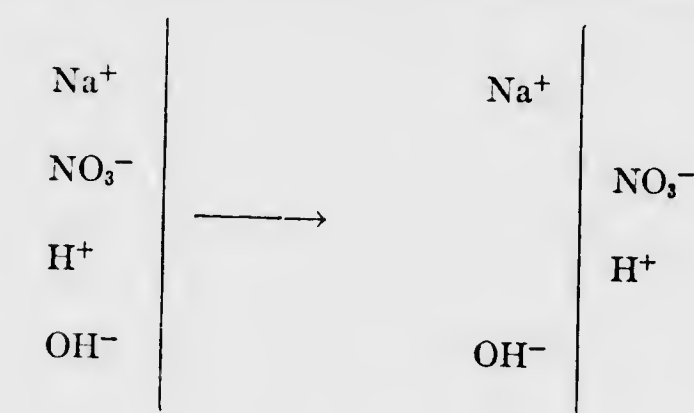
DEGREE OF ACIDITY	CHARGE ON ROOTS	SALT APPLIED
Slightly acid, neutral or alkaline.....	-	$\underline{NH_4^+} > SO_4^{--}$
	-	$\leftarrow Na^+ > NO_3^-$
Distinctly acid, pH 5.5 and below.....	+	$\leftarrow SO_4^{--} > NH_4^+$
	+	$\leftarrow NO_3^- > Na^+$

where underlining indicates the ion preferentially absorbed, and $>$ indicates the rate of absorption is greater than.

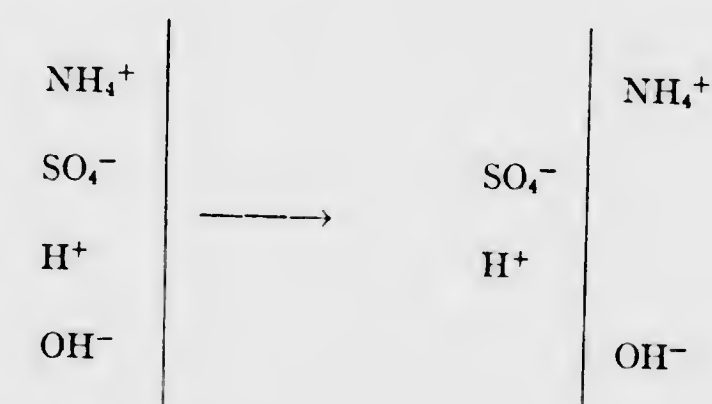
The fact that in alkaline, neutral, or slightly acid media cations exert the dominant influence in absorption, and conversely that in acid media the anions are the dominant factor explains the superiority of ammonium sulfate

over sodium nitrate under the former conditions and of sodium nitrate over ammonium sulfate under the latter. The physiological effect of ammonium salts will accordingly become relatively weaker as the H^+ -ion concentration *increases*, whereas the physiological effect of sodium nitrate, on the other hand, will be weaker as the H^+ -ion concentration of the soil *decreases*.

The influence of membrane hydrolysis on the "selective" absorption of ions.—It can be shown from thermodynamic principles that an ion cannot permeate the cell membrane without an oppositely charged ion (17). If a dialyzable electrolyte is present on one side of a semi-permeable membrane and on the other pure water, a hydrolytic decomposition of the salt occurs. Using Na^+ as the cation and NO_3^- the anion the process may be illustrated thus:



The median will, therefore, tend to become alkaline because of the much slower rate of penetration of Na^+ compared with NO_3^- . Na^+ is much more strongly hydrated than NO_3^- . On the other hand, if $(NH_4)_2SO_4$ is used the medium will tend to become acid because of the much greater rate of penetration of NH_4^+ compared with SO_4^{--} . SO_4^{--} is more strongly hydrated than NH_4^+ . Thus:



This hydrolytic action due to the presence of the membrane is promoted in accordance with the Gibbs-Donnan law by the difficultly permeable Na^+ ions in the one case and the SO_4^{--} ions in the other.

These interpretations are supported by the results of numerous experiments. For example, Prianishnikov (70) found that sugar beet and maize grown in sand cultures, with frequent renewal of the culture solutions so as to maintain practically constant reactions, gave much better results with ammonium salts than with nitrate salts at pH 7.0 but much poorer results with ammonium salts than with nitrate salts at pH 5.5. And Hager and Stollenwerk (17) found

that the ratio of nitrogen to sulfate $\left(\frac{N}{SO_4}\right)$ absorbed from ammonium sulfate by rye, oats, wheat, and barley ranged from 3.06:1 for rye to 5.4:1 for oats.

On the old fertility plots of the Pennsylvania State College the response from ammonium sulfate was better than from sodium nitrate during the first decade but afterwards, as the hydrogen-ion concentration of the soil of the ammonium sulfate plots increased, the response from sodium nitrate was much better than from ammonium sulfate. The pH range of the ammonium sulfate plots is at present (tier I) 4.18-4.50 and of the sodium nitrate plots 5.51-5.71.

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FACTORS INFLUENCING THE VITAMIN CONTENT OF FOODS

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THE PENNSYLVANIA STATE COLLEGE
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Factors Influencing the Vitamin Content of Foods¹

R. ADAMS DUTCHER

IN SPITE of the fact that scarcely 20 years have elapsed since the pioneer work of Hopkins, Funk, Holst and Frölich, McCollum, and Osborne and Mendel which led to the inception of the vitamin hypothesis, a very extensive literature has accumulated relative to vitamins and deficiency diseases. When we attempt to review the research literature relative to the various factors which influence the vitamin content of foodstuffs we are often impressed by the apparent lack of agreement among workers in certain phases of vitamin research and the paucity of authentic data in many other lines of vitamin investigations. It is difficult, therefore, to attempt to make a satisfactory resumé of the literature in this field of research for the reasons just enumerated and also for the reason that our methods are in a state of flux due to the increasing evidence for the existence of new vitamins and the apparent multiple nature of those which had been recognized heretofore as individual substances.

The following system of vitamin nomenclature, first suggested by McCollum, will be used in referring to the various accessory food factors:—

(1) **Vitamin A.** The fat-soluble factor, the absence of which causes experimental animals to lose their capacity to grow; which produces a type of sterility and causes the development of xerophthalmia and the apparent lowering of resistance to certain types of infections especially infections of the respiratory tract.

(2) **Vitamin B.** The heat-labile antineuritic fraction of the old vitamin B complex, the absence of which leads to the development of experimental beriberi.

(3) **Vitamin C.** The antiscorbutic vitamin, the etiological factor in the development of experimental scurvy.

(4) **Vitamin D.** The fat-soluble vitamin which has to do with the efficient utilization of calcium and phosphorus and which, when absent from the diet, is an etiological factor in the causation of rickets. This vitamin is, without doubt, identical with activated ergosterol.

(5) **Vitamin E.** The fat-soluble fertility factor, the absence of which brings about sterility in experimental animals. A discussion of this vitamin will be omitted due to the fact that there is no evidence at present to show that this factor is of clinical importance in human or domestic animal nutrition.

(6) **Vitamin G.** The heat-stable fraction of the old vitamin B complex which stimulates appetite and, therefore, growth, and which, when absent from the diet, leads to the development of pellagra-like skin lesions.

¹The material in this bulletin is based on a De Lamar Lecture delivered at Johns Hopkins University, January 27, 1931.

Since the fractionation of what was formerly known as vitamin B is of comparatively recent date, little information is available regarding the distribution of vitamins B and G, which are known in British terminology as vitamin B₁ and vitamin B₂, respectively. The term "vitamin B complex" will be used, therefore, when it is impossible to refer to the individual components, B and G.

For convenience of discussion the subject of this bulletin is divided into two main topics, viz., (1) **Factors Influencing the Natural or Normal Vitamin Content of Fresh Foods**, and (2) **The Influence of Food Treatment on Vitamin Potency**.

1. FACTORS INFLUENCING THE NATURAL OR NORMAL VITAMIN CONTENT OF FOODS

While there are exceptions to the general rule that vitamin synthesis is a property peculiar to plants and that animals merely possess the power of vitamin storage, it will be convenient to discuss our natural foods under two general headings, viz., (a) Foods of Plant Origin, and (b) Foods of Animal Origin.

(a) Foods of Plant Origin

Lack of space prevents detailed reference to the literature concerning vitamin distribution in plants, other than to state that air-dry seeds and cereals as a group are deficient in vitamins A, C and D. McCollum, Simmonds and Pitz (1) have pointed out certain exceptions to this general statement, viz., that flax and millet seeds contain appreciable amounts of vitamin A. Sure (2) has made similar observations on the Georgia velvet bean and Steenbock (3) was the first to emphasize the value of yellow corn as a source of vitamin A.

The value of cereals, however, as a source of the vitamin B complex has been recognized for many years. Eijkman (4) was the first to point out that whole rice was curative in experimental beriberi, while the disease could be produced at will with polished rice. Subsequent observations showed that very few cases of beriberi could be found in communities where red or brown rice was a staple article of diet, while milled or white rice tended to cause an increase in the number of people afflicted with this disease.

Comparison of seed structures (5) has shown that the B complex is largely residual in the embryo or germ which explains the obvious vitamin deficiency of highly bolted milled cereals. When seeds are allowed to germinate in the dark, vitamin C is increased (6) but the amount of vitamin A is not increased appreciably (7). If the sprouting of seeds occurs in the light with the subsequent formation of pigment in the normal seedling, it is found that vitamin A potency has increased.

As a result of such studies it has been thought that vitamin synthesis is correlated with the metabolic processes of the plant. This theory has received support from the fact that the leaf, which is the most ac-

tively metabolizing tissue in the plant, is invariably richer in vitamins A, B, C, D and G than any other plant tissue. When the plant matures, however, and seeds are formed, the vitamin B complex is stored in the seed.

A few scattered attempts have been made to study the effect of fertilizer treatment on the vitamin content of the seed. Rowlands and Wilkinson (8) showed that grass seeds, grown on soils fertilized with farm manure, were superior in their content of the vitamin B complex to seeds grown on soils treated with artificial fertilizer, which confirms the findings of McCarrison (9). Hunt (10) could find no difference between spring and winter wheats. After four years of experimentation he obtained some evidence to show that a phosphatic fertilizer seemed to increase the B complex content of wheat. Since phosphorus is known to stimulate the efficiency of seed formation in plants, Hunt's conclusions appear to be sound.

Dye and Crist (11) working with lettuce, grown in soils fertilized with sheep manure and combinations of potassium nitrate, mono-calcium phosphate and potassium chloride, obtained evidence that synthesis of vitamin A in the leaf of the lettuce plant was related to the size and greenness of the plant and that the addition of sheep manure alone caused some increase of vitamin A, while the addition of a complete inorganic fertilizer produced results superior to those obtained with sheep manure. These investigators call attention to the fact that a definite correlation exists between chlorophyll deposition (greenness) and vitamin A content.

Reference has been made to Steenbock's early observations (3) which were advanced to support the hypothesis that vitamin A is either identical with carotin or very closely associated with the carotinoid pigments. His work and the work of others showed that carotinoid pigments and vitamin A potency seemed to go hand in hand. Palmer and co-workers (12) and others showed, however, that there were exceptions to this general rule and that it was clear that these substances were not identical and that their association was probably fortuitous.

Recent work by Euler (13), Moore (14), Capper (15) and Drummond and coworkers (16) furnished a satisfactory explanation for the diverse interpretations of Steenbock and Palmer. Recent investigations show that pure crystallized carotin possesses properties characteristic of vitamin A, when fed to experimental animals. Light absorption spectra of purified carotin and of pigment-free preparations of vitamin A, differ markedly. It would appear, therefore, that carotin and vitamin A are not identical.

Liver oils of vitamin A-deficient animals do not show spectral lines characteristic of vitamin A. When, however, animals have been fed sufficient recrystallized carotin to bring about the typical physiological response, the liver oils show spectral lines which are characteristic of vitamin A. These data lend support to the hypothesis that carotin is the parent substance or precursor of this vitamin.

The hypothesis that the liver is the seat of transformation of carotin to vitamin A has been confirmed by Oleott and McCann (17). These workers were able to show that purified carotin may be changed to vitamin A outside the animal body through the influence of liver extracts. Since the liver extracts do not possess vitamin A synthesizing power after they have been heated, these authors have advanced the hypothesis that the liver owes its synthetic power to an enzyme which they have named *carotenase*.

It is probable that we shall find that some of our so-called "vitamin A-rich" foods are really quite deficient in this vitamin, per se, but that these foods are really rich in the parent substance, carotin. From nutritional and clinical standpoints this fact need not cause concern, so long as the fat-soluble factor is rendered available for the animal organism.

Coward (18) showed that vitamin A synthesis in plants is associated with lipochrome formation and that light is necessary for the production of this vitamin. Widmark (19) submits evidence to show that plants lose their ability to synthesize vitamin A when they lose their power to form chlorophyll and lipochrome pigments. This hypothesis is supported by the fact that green leaves of lettuce and of cabbage are superior in vitamin A content to the pigment-free inner leaves.

We have found, in our own laboratory, that chlorotic spinach (grown on manganese-deficient soil) contains much less vitamin A potency than spinach which has a normal deposition of chlorophyll. The work of Hess and Unger (20) and of Osborne and Mendel (21) indicates that the vitamin content of carrots and of alfalfa is at its maximum during the early stages of growth, when the metabolic processes are most active.

It would appear that the tubers and fleshy roots such as the carrot, beet, potato and parsnip are analogous to the seed, i. e., they serve largely as storage organs for the vitamins which are synthesized in the leaf. Such foods are usually good sources of the vitamin B complex and of vitamin C, but vary in their content of vitamin A, the pigmented roots such as carrots and sweet potatoes being good sources of this vitamin, while white potatoes and white parsnips are deficient in this factor.

The fruits also serve as storage organs and are usually excellent sources of the water-soluble vitamins but vary in their value as a source of vitamin A. The tomato and citrus fruits, particularly oranges and lemons, are among our best sources of vitamin C, while the pigmented tomato is also unusually potent when fed as a source of vitamin A.

Clow and Marlatt (22) have published data confirming, in a measure, the work of House, Nelson and Haber (23) relative to the synthesis of vitamin C in the tomato. Both groups agree that ethylene-ripening neither hinders nor enhances vitamin synthesis. Greenhouse tomatoes ripened on the vine were not quite so potent, as a source of vitamin C, as field ripened tomatoes. Ripening after removal from the vine did not seem to alter or affect the development of vitamin C. Bracewell and

Zilva (24) state that the vitamin C content of oranges seems to be the same whether the fruit is picked at the beginning or at the end of the season. These writers were unable to find that cultivation, origin of stock, age of tree or nature of soil had measurable effects on the amount of vitamin C in oranges. It was impossible to correlate antiscorbutic potency with total solids or hydrogen-ion concentration and after two months storage at 15° Centigrade no appreciable loss of vitamin C could be detected.

Hauge and Trost (25) concluded that the vitamin A content of yellow corn can be correlated with the yellowness of the endosperm. The color of the pericarp did not seem to be a factor of importance. These authors also concluded, as a result of cross-pollination studies, that the vitamin A content of corn is controlled by ordinary hereditary factors and that the genes are the same as those governing the development of the yellow endosperm. In the light of the new work on the function of carotinoid pigments, it is probable that these writers were dealing almost entirely with the inheritance of pigmentation and not of vitamin A, per se.

(b) Foods of Animal Origin

From the standpoint of the physiologist and the nutrition worker, the vitamin content of foods of animal origin is of two-fold interest. Not only are these foods of interest from the standpoint of their value as sources of the vitamins but their variability, relative to the capacity to store certain of these accessory factors under various conditions, presents a most interesting problem.

The theory that vitamins cannot be synthesized in the animal body but must be furnished preformed in the food has received support from the accumulated evidence that animals develop vitamin deficiency diseases when forced to subsist on vitamin-free rations. If the various vitamins are added to the diet of such vitamin-deficient animals, the symptoms of the respective diseases gradually disappear and the experimental animals tend to deport themselves in a normal manner. Some workers have contended, therefore, that the animal organism is incapable of vitamin synthesis.

When, however, foods of animal origin are studied by biological-response methods, it is found that certain types of foods, such as liver, kidney, milk and eggs, may be unusually potent sources of certain vitamins. Certain other tissues, of which lean muscle tissue may be considered representative, are much less potent sources and often may be considered vitamin-deficient. On the other hand, liver, milk and eggs have been found to vary markedly in vitamin content. As a result, investigators have endeavored to determine the reasons for the variations just described.

Owing to the importance of cod liver oil as a source of vitamins A and D, it is to be expected that science should turn her attention to the origin of the vitamins stored in the liver of the cod fish. Most re-

searches to date (26) indicate that the vitamin content of fish roe and fish livers has its source in the microscopic chlorophyllous marine plants which develop in the spring, coincident with the increase in light intensity. In the case of the cod fish, it is thought that this increase in marine plant life is associated with the observed increase of microscopic animal life, particularly the copepods which, in turn, are undoubtedly associated with the health and well-being of young fish and other marine animals, which depend upon these organisms for food. It seems reasonable to postulate that the vitamins stored by these small fish are finally stored in the livers of the cod and other larger fish, since the former serve as food for the latter.

Variability in cod liver oils has been attributed to variations in vitamin synthesis by marine plants, due to abnormal seasonal conditions; to variations in the food supply and to the physical condition of the cod fish at the time it is caught.

Bills (27) contends, however, that it is impossible for the cod fish to eat sufficient food to account for the unusual content of vitamins A and D in cod liver oil. He believes that the cod fish is able to synthesize vitamins to an appreciable extent.

Funk (28) was the first to suggest, in 1913, that a definite relationship exists between the vitamin content of milk and the food ingested by the lactating animal. Three years later McCollum and coworkers (29) offered the first experimental evidence that vitamins A and B (complex) pass into the milk only as they are present in the diet of the mother. These investigators, working with rats, were unable to make actual measurements of the vitamin potency of rats' milk, but based their statements on the ability of the mothers to rear their young.

In August, 1919, Barnes and Hume (30) and Dutcher, Pierson and Biester (31) announced simultaneously that they had noted seasonal variations in the nutritive value and antiscorbutic properties of cow's milk. As a result of these observations, Dutcher and coworkers (32) conducted a series of feeding experiments in which two cows were fed (for about four months) on a ration which was vitamin deficient. During this period guinea pigs were placed on experiment from time to time. These animals received a scurvy-producing diet, and varying quantities of the theoretically vitamin-deficient cow's milk were fed as the source of the antiscorbutic factor. In June the cows were given access to luscious green pasture and the experiment was continued. At the point where the cows were sent out to pasture, a number of guinea pigs were in various stages of scurvy as a result of the vitamin deficient milk. These animals showed definite improvement almost immediately and new animals, added month by month, grew well and failed to show scorbutic symptoms on daily allotments of "green grass milk" from the same cows.

It was concluded that the "vitamin content of milk is dependent upon the vitamin content of the ration ingested by the cow"; that 20 c. c. of "summer milk" were superior in nutritive value and antiscor-

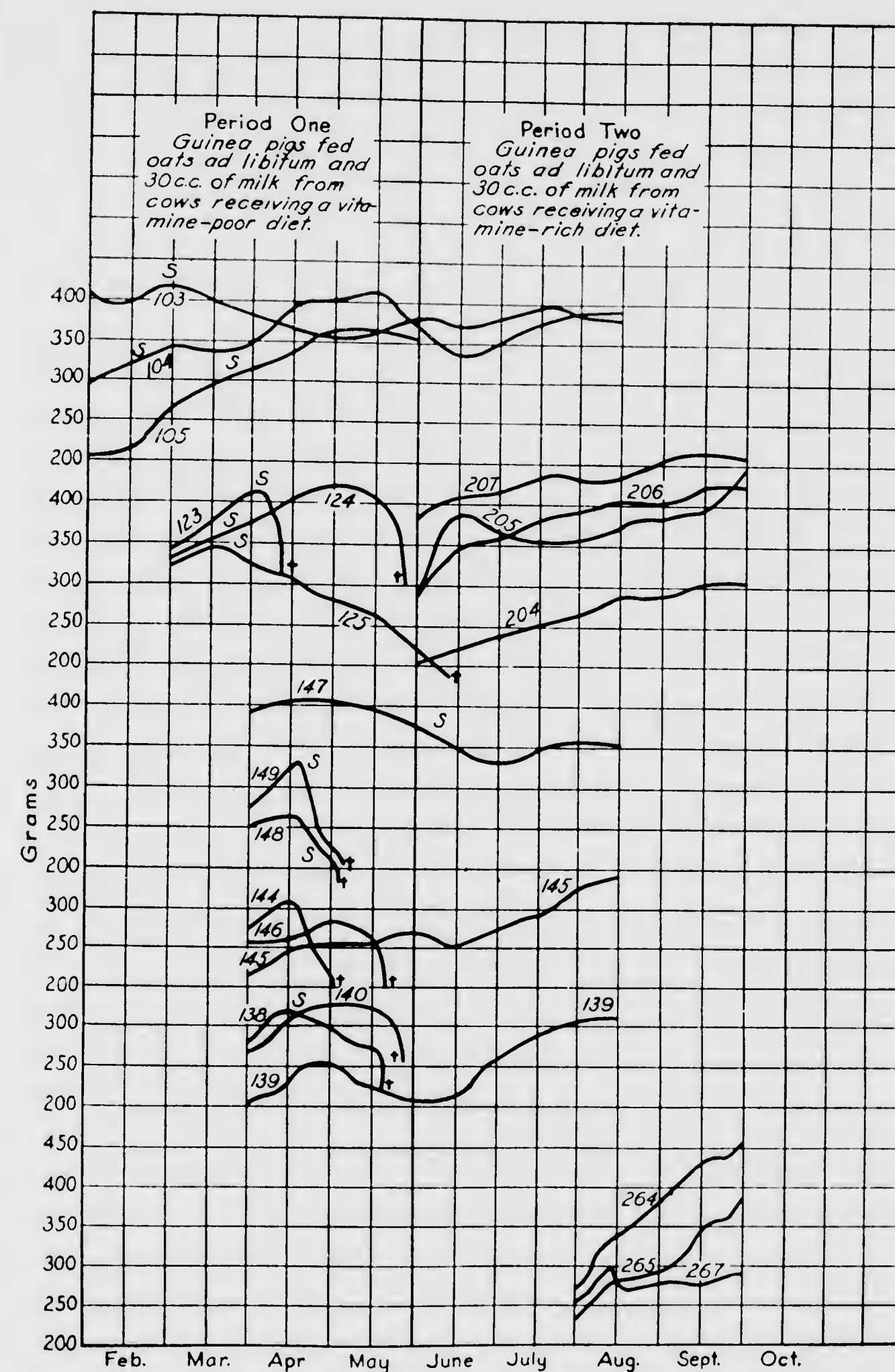


FIG. 1. SHOWING THE INFLUENCE OF THE DIET OF THE COW ON THE AMOUNT OF THE ANTISCORBUTIC FACTOR, VITAMIN C, FOUND IN COW'S MILK.

When cows are fed a vitamin-deficient ration, there is a tendency for the milk to become vitamin-poor rather slowly. When vitamin-rich rations are fed, there is a tendency for the milk to become vitamin-rich rather rapidly. (Data of Dutcher et al.) (Courtesy of the Journal of Biological Chemistry.)

butic properties to 60 c. c. of "winter milk"; that the milk became poor slowly when the cow's ration was low in vitamins, but it tended to become vitamin-rich rapidly, following the enrichment of the diet. These experiments were corroborated by other investigators (33).

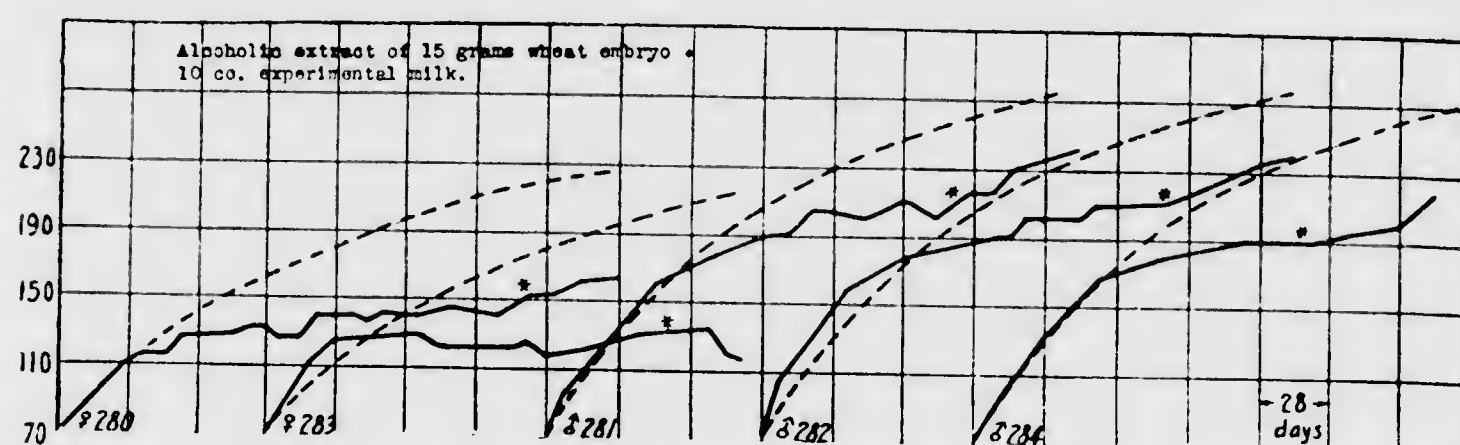


FIG. 2. SHOWING GROWTH OF RATS WHEN THE VITAMIN B COMPLEX WAS FED IN THE FORM OF WHEAT EMBRYO EXTRACT AND VITAMIN A WAS FURNISHED BY 10 CC. (DAILY) OF "WINTER MILK."

Note that all growth curves are sub-normal.

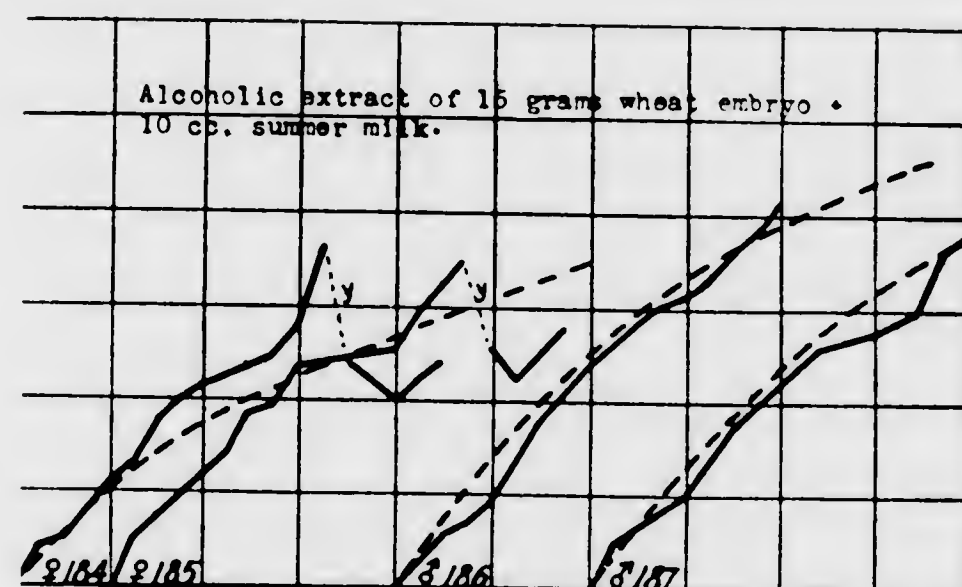


FIG. 3. SHOWING GROWTH OF RATS WHEN THE VITAMIN B COMPLEX WAS FED IN THE FORM OF WHEAT EMBRYO EXTRACT AND VITAMIN A WAS FURNISHED BY 10 CC. (DAILY) OF "SUMMER MILK."

The broken curves at "y" indicate that young were born at these points. (Data of Kennedy, Dutcher and Eckles.) (Courtesy of the Journal of Biological Chemistry.)

In 1922 Kennedy, Dutcher and Eckles (34) published the first quantitative evidence that the vitamin A content of milk was also dependent upon the diet. The milk used in these experiments came from the same cows that were used in the antiscorbutic work. Butter fat was studied in addition to the raw milk. It was concluded that vitamins A and B were subject to the same rule that had been proposed for vitamin C. It was impossible to show marked fluctuations, however, in the vitamin B complex and, at the time, we attributed the rather constant vitamin B content of milk to the fact that we could not feed a vitamin B free ration to the cows, owing to the necessity of feeding some whole grain.

More recently, we (35) have found an additional explanation for the rather constant amount of the B complex in cow's milk. Dr. S. I. Beechdel, of the Department of Dairy Husbandry, observed that heifer calves grew normally to maturity on rations that were deficient in the vitamin B complex. In fact, he was able to obtain three generations of animals on a diet which was so deficient in the B complex that rats would not grow on this ration unless yeast was supplied. A study of the milk of these cows showed that the B complex was present in normal amounts, while, according to previous work with the other vitamins, the milk should have been deficient in this complex. It was evident that the cow was synthesizing her own supply of the B complex, since the milk was practically normal in this regard. Since the cow is a ruminant, it seemed reasonable to believe that she might possess the ability to synthesize vitamins through the propagation of microorganisms in the rumen or storage stomach.

By means of a permanent fistula in the rumen, it became possible to study the vitamin-B-complex free food at intervals following deglutition (swallowing) and preceding the passage of this food from the rumen into the other stomachs.

Feeding trials showed that alcoholic extracts of the fermented feed contained the vitamin B complex. Subsequent studies by Professor M. H. Knutsen revealed a surprisingly large microflora consisting almost wholly of a bacterium belonging to the *Genus Flavobacterium*. This organism was grown on media free from the B complex and the bacterial cells were isolated, dried and fed to rats and were found to be potent in the vitamin B complex. As a result, it was concluded that cattle are capable of synthesizing the vitamin B complex in the rumen through the agency of microorganisms. Professor Knutsen has named this organism *Flavobacterium vitarumen*. Damon and others (36) have shown that other microorganisms also possess the property of synthesizing the vitamin B complex.

While the ruminant may be able, under such conditions, to obtain the vitamin B complex through the agency of bacteria, there is little or no evidence that the vitamins can be synthesized by the tissues. We have already cited evidence supporting the theory that vitamin A can be synthesized, by the liver, from the carotinoid pigments. Another exception seems to exist in the case of rats and birds in so far as their needs for vitamin C are concerned. All evidence points to the fact that these two types of animals can live and grow without the antiscorbutic factor. That the rat can actually synthesize vitamin C seems to have been proved quite conclusively by Parsons (37), who reared rats on a vitamin C-free diet for 15 months. She was able to show that the livers from these rats contained sufficient vitamin C to cure acute scurvy in guinea pigs.

Further proof (38) that the rat differs markedly from the guinea pig was obtained by curing acute scurvy by feeding the livers of normal guinea pigs. The livers of guinea pigs afflicted with scurvy, however, had no curative affect when fed to other scorbutic animals. It is

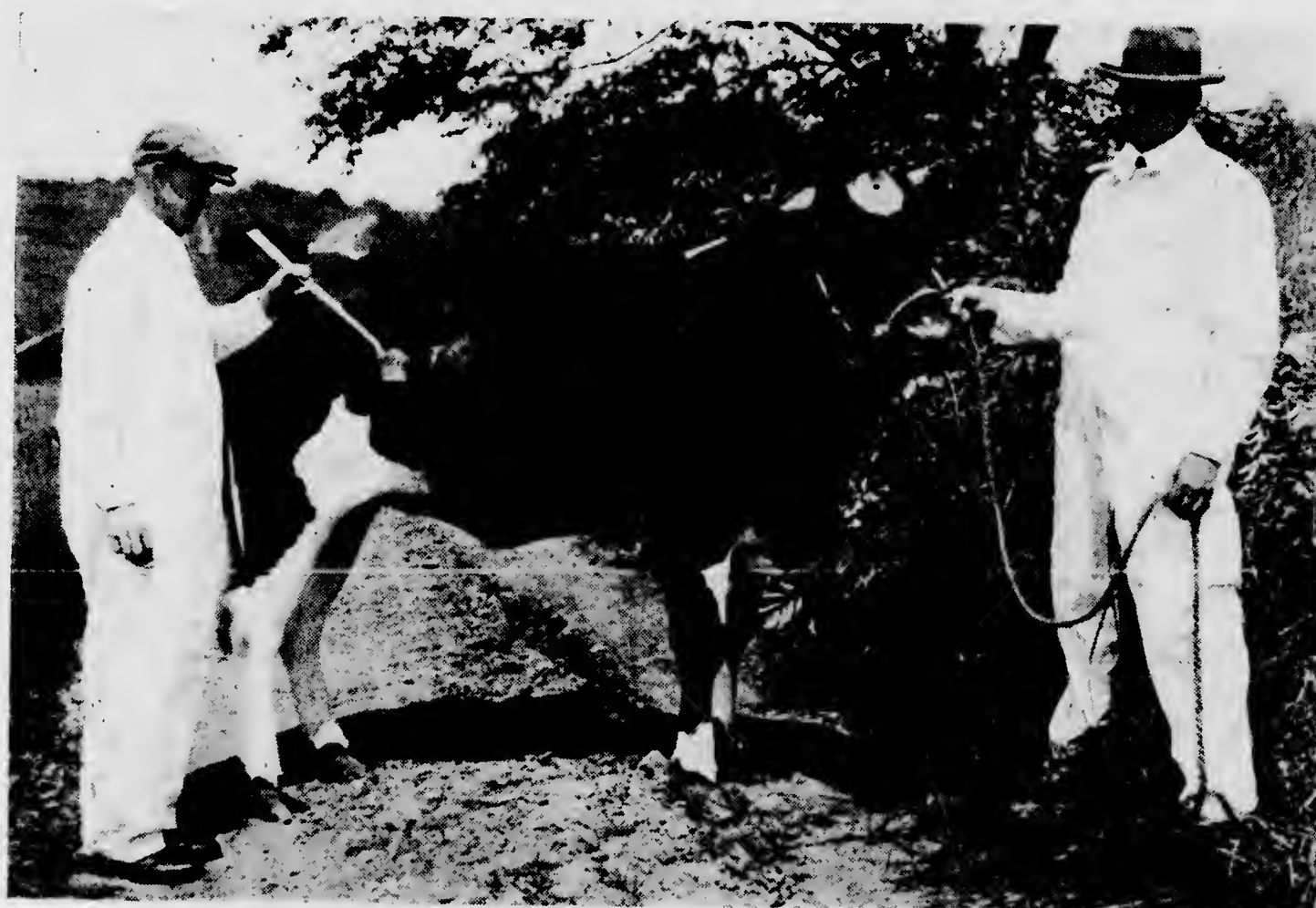


FIG. 4. PENN STATE HOMESTEAD JESSIE, SHOWING PERMANENT FISTULA IN THE RUMEN FROM WHICH SAMPLES OF THE RUMEN CONTENTS WERE REMOVED AND STUDIED.

It was found that the rumen harbored an organism (*Flavobacterium vitarumen*) which possessed the power to synthesize the vitamin B complex. (Data of Bechdel, Dutcher, Honeywell and Knutsen.)

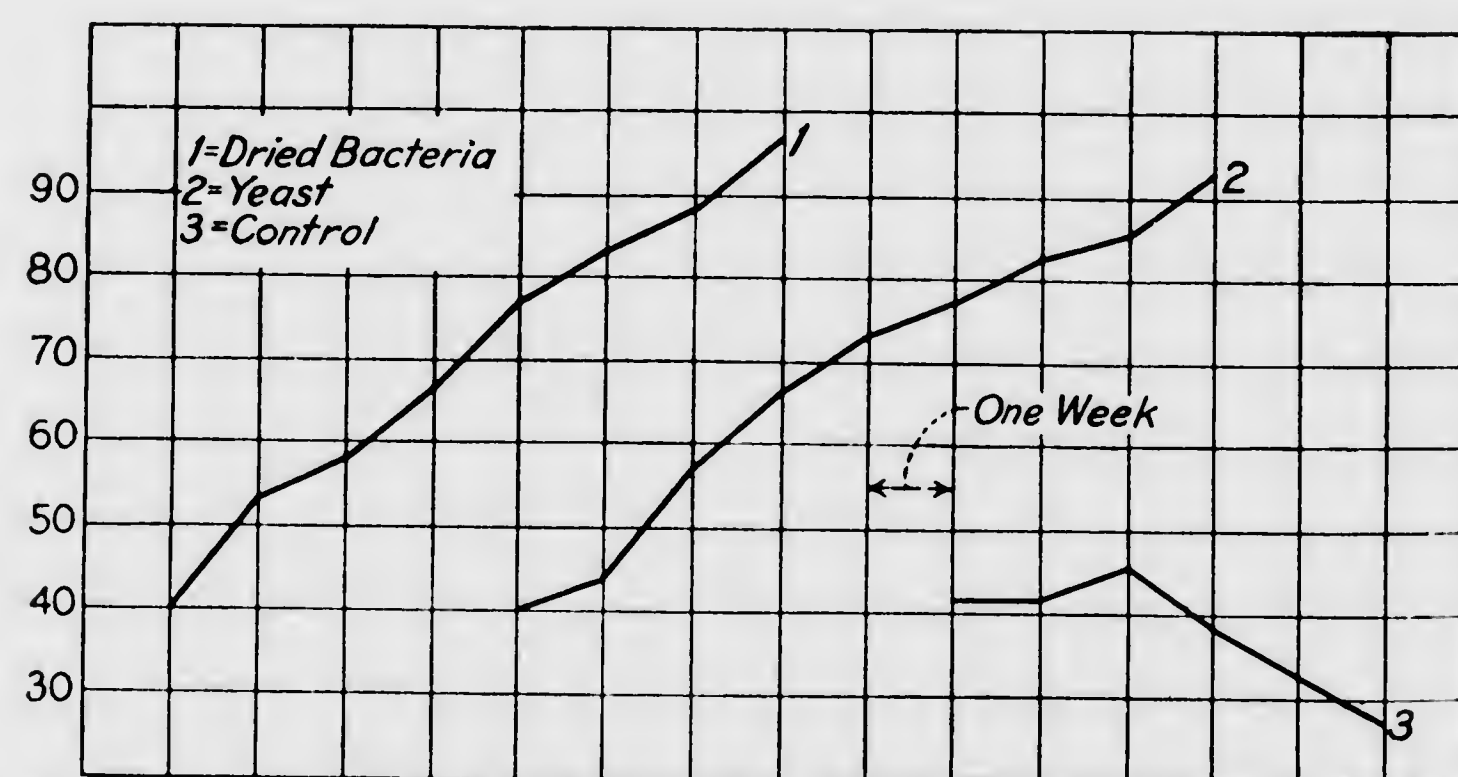


FIG. 5. GROWTH CURVES OF GROUPS OF RATS WHICH RECEIVED A BASAL RATION WHICH WAS DEFICIENT IN THE VITAMIN B COMPLEX.

Group 3 is the control group which received no supplement. Group 2 received daily supplements of yeast known to be rich in the B complex. Group 1 received daily supplements of dried *Flavobacterium vitarumen* which had been isolated from the rumen of the cow shown in Fig. 4.

not impossible that some of the higher domesticated animals are also capable of synthesizing the antiscorbutic factor in the tissues.

Carriek and Hauge (39) have submitted evidence to show that domestic poultry possess the power to synthesize vitamin C and that livers of birds, fed on diets deficient in this vitamin, are capable of curing scurvy in scorbutic guinea pigs. While the experimental data are meagre, it would appear that the vitamin content of eggs varies directly with the vitamin content of the diet of the hen (40).

All the known vitamins are stored in the liver and to a less degree in other body tissues. Vitamin D, like vitamin A, is stored mainly in the liver fat. This phenomenon (vitamin storage) is of importance from the standpoint of the newly born young. If the mother is able to store adequate amounts of vitamins during the period of pregnancy, the young are almost certain to have a better physical and physiological "start in life" than the young which are born from poorly nourished mothers. This has been emphasized in a most instructive manner by Sherman and coworkers (41).

No systematic work has been done to study the factors influencing the vitamin D content of our natural plant foods. It seems logical to believe that the amount and intensity of sunlight are most important factors, since there is evidence to support the postulation that vitamin D arises through the irradiation of the natural sterols in the plant tissues during the growing and harvesting periods.

A number of successful experiments have been recorded in which milk, from cows irradiated with ultra violet light, has been shown to be more highly antirachitic than milk from the same cows before the light treatment (42). We (43) have tested butter fat from cows subjected to sunlight and found that the antirachitic potency was somewhat higher than butter from cows which were fed in the dark.

Steenbock, Hart and coworkers (44) made similar observations on goats milk but were unable to find that irradiation of the cow affected the antirachitic potency of the milk, which substantiates the work of Luce (45), who concluded that sunlight is not an important factor. Chick and Roseoe (46), however, believe that sunlight is more important than diet so far as the vitamin D content of cows' milk is concerned. Golding and associates (47) have shown that the antirachitic potency of milk can be enhanced if the cow receives appreciable amounts of cod liver oil. Hart and coworkers (48) were able to increase the antirachitic potency of cows' milk by feeding irradiated yeast.

With this brief resumé regarding some of the factors influencing the natural variations in foodstuffs, we shall now consider some of the factors which tend to destroy or preserve vitamins when foodstuffs are subjected to various types of food treatment.

II. Food Treatment and Vitamin Potency

For convenience of discussion we shall consider food treatment under the following headings:—(a) Harvesting, (b) Storage, (c) Heat treatment, and (d) Irradiation.

(a) Harvesting and Field Factors

It has been pointed out that the vitamin content of leafy plants and vegetables may be correlated with greenness, metabolic activity and maturity. Old, mature tissues tend to be less valuable as sources of vitamins (particularly A and C) than the young rapidly metabolizing tissues.

In 1925 Steenbock, Hart, and associates (49) stated that clover hay, which had been bleached by long exposure to sunlight, rain and dew, was less valuable as a source of vitamin A than clover hay which had been dried more quickly and in a manner which tended to preserve its green color. The same hay, on the other hand, possessed enhanced calcifying properties when it was subjected to sunlight during the curing process, while the antirachitic potency was less satisfactory when the hay was dried under eaps or in the attic of the barn.

In a recent paper, Russell (50) has corroborated the results just described and states that alfalfa, dried artificially in a modern hay drier, was about seven times as potent in vitamin A as the same material dried and cured by the usual field methods. He also noted an increase in the content of vitamin D when the hay was dried in the sun. Practical feeders have observed that bright alfalfa harvested during the rapidly growing period just prior to blossoming, and properly dried and cured, is superior in nutritive value to the more mature hays and to those which have been bleached during the drying process.

Hauge and Aitkenhead (51) have studied the effect of artificial drying versus natural curing of hays and conclude that much, if not most, of the destruction of vitamin A is due to enzymatic changes during slow drying in the field. When enzymatic changes were reduced to a minimum (by heat) it was found that vitamin A was preserved very efficiently in spite of the presence of hot air and the relatively high temperatures of the artificial drier.

It is evident that the environmental factors which encourage fermentation and oxidative changes are to be avoided if vitamins A and C are to be preserved.

(b) Storage

It is recognized, quite generally, that the vitamin content of fresh vegetables and fruits becomes depleted during storage (52). The rapidity of vitamin destruction in fruits and vegetables during storage is due primarily to the oxidative changes which occur during the normal respiration of the plant tissues. Such factors as temperature, hydrogen-ion concentration, enzymatic activity, etc., are of vital importance. Any environmental condition which will reduce tissue respiration to a minimum should tend to preserve vitamin potency.

Other foodstuffs such as butter and cod liver oil also show progressive loss of vitamin A during storage. This can be prevented to some extent by storing these foods in an atmosphere of hydrogen gas in order that oxidation can be reduced to a minimum.

Quinn, Hartley and Derow (53) have published a recent paper in which they have shown that dried spinach is susceptible to a loss of about 70 per cent of its vitamin A during a storage period of 12 to 15 months. They noted that the stored spinach had lost some of its characteristic green color during this period. This destruction is due, undoubtedly, to the action of atmospheric oxygen on the carotinoid pigments.

The presence of rancid fat has been shown to stimulate the destruction of vitamin A. It is quite evident that some of the constituents of rancid fats possess a catalytic effect on auto-oxidative changes during storage. Oxygen, which is necessary for these changes, may be furnished through the decomposition of naturally occurring organic compounds as well as from the atmosphere.

Holm (54) believes that unsaturated fats or their peroxide-like decomposition products have a catalytic effect on the destructive oxidative processes which occur in rancid foods.

Iron salts (55) which play a part in biological oxidations have been shown to hasten vitamin A destruction during storage, while Hess (56) has shown that copper salts may hasten the destruction of vitamin C in heated milk.

While very little work has been done relative to the effect of long-time storage of frozen food products, it would appear that the most susceptible vitamins are fairly well preserved. Jones, Murphy and Moeller (57) report that frozen eggs which were stored for nine years had suffered little, if any, loss of vitamin A. There is no evidence that vitamins B or D suffer appreciable loss during ordinary storage. Seeds which have been stored for long periods often retain appreciable amounts of the vitamin B complex, provided respiration is kept at a minimum.

Hydrogen-ion concentration is a factor of great importance so far as the preservation or destruction of vitamins B and C is concerned. Acidity is necessary for preservation, while alkalinity possesses a destructive effect. The presence of natural or artificial anti-oxidants of the type of hydroquinone (58) has been shown to prevent or delay vitamin destruction during storage.

(c) Heat Treatment

When we consider the effect of heat on the vitamin content of foods we are confronted with a voluminous literature, much of which is contradictory and obscure. It is impossible to review this literature in detail, but it will be helpful to emphasize some of the researches which have led to a better understanding of the conditions under which foodstuffs may be treated or processed in order that their vitamin content may be preserved.

(1) Desiccation and Fermentation

If plant tissues are dried rapidly at relatively high temperatures to insure the inactivity of enzymes, it is found that vitamins A and C are preserved much more efficiently than when the drying process is conducted over longer periods at lower temperatures. If it is possible to eliminate atmospheric oxygen the amount of original vitamin preserved will be even greater. Miss Whittemore and coworkers of the Rhode Island Experiment Station have prepared unusually potent dried spinach by drying the leaves in an atmosphere of hot carbon dioxide and carbon monoxide at a temperature which insures relatively rapid drying. These samples have been tested in our laboratory.

In 1918, Delf and Skelton (59) reported that the antiscorbutic factor was almost completely destroyed during the slow dehydration of cabbage. During the same period, Givens and McClugage (60) made the first semi-commercial studies relative to the effect of drying on tomatoes and oranges. These writers concluded that the antiscorbutic properties of dried tomatoes and dried oranges were high, in spite of the fact that some destruction of vitamin C might have taken place.

Ellis, Steenbock and Hart (61) were among the first to make a systematic study of the factors influencing the stability of vitamin C. These writers reported that fresh cabbage dried for 35 hours in a vacuum at 65° C. had lost its antiscorbutic properties. There is little question but that this destruction was due to enzyme action and intra-cellular oxidation, since the drying process undoubtedly allowed considerable time to elapse before enzyme destruction took place. They found also that sauerkraut had lost some of the antiscorbutic potency present in the fresh cabbage. They believed this destruction to be due to the oxidative changes during fermentation, although they stated that there was no way of telling (at that time) how much of the destructive effect was due to heat.

Owing to the fact that sauerkraut had been used successfully as an antiscorbutic food on long sea voyages and, since some clinicians were convinced of its value in this regard, the problem was re-investigated by Clow, Marlatt, Peterson and Martin (62), a report of which was published in 1929. These writers found that fresh sauerkraut is about one half as potent as raw cabbage. They express the belief that the destruction of vitamin C is due to oxidation during the fermentation process. They call attention, however, to the fact that their work does not warrant the assumption that all commercial bulk or canned sauerkraut will contain vitamin C.

The important question concerning the vitamin content of dried or desiccated milk has received considerable attention. From the writer's personal experience and from the available literature it would appear that cow's milk may be desiccated satisfactorily by spray or roller processes if proper precautions are taken. Jephcott and Bacharach (63) report that dried milk made by their roller process was superior, as a source of vitamin C, to dried milk made by the spray process. Hess, Unger and Supplee (64) have published data showing that roller pro-

cess powders retained adequate amounts of vitamin C. Cavanaugh, Dutcher and Hall (65) compared spray process dried milks with the original raw milks from which the powdered milks were made and concluded that the spray method, of itself, was not a destructive factor, since the raw and desiccated milks did not differ appreciably in antiscorbutic potency.

Whole milk powders, made by both processes, have been tested in our own laboratory and have been found to contain ample but variable amounts of vitamin A. It cannot be assumed, however, that all desiccated milks on the market are satisfactory sources of vitamins A and C for the reason that raw milk is variable in these vitamins and careful

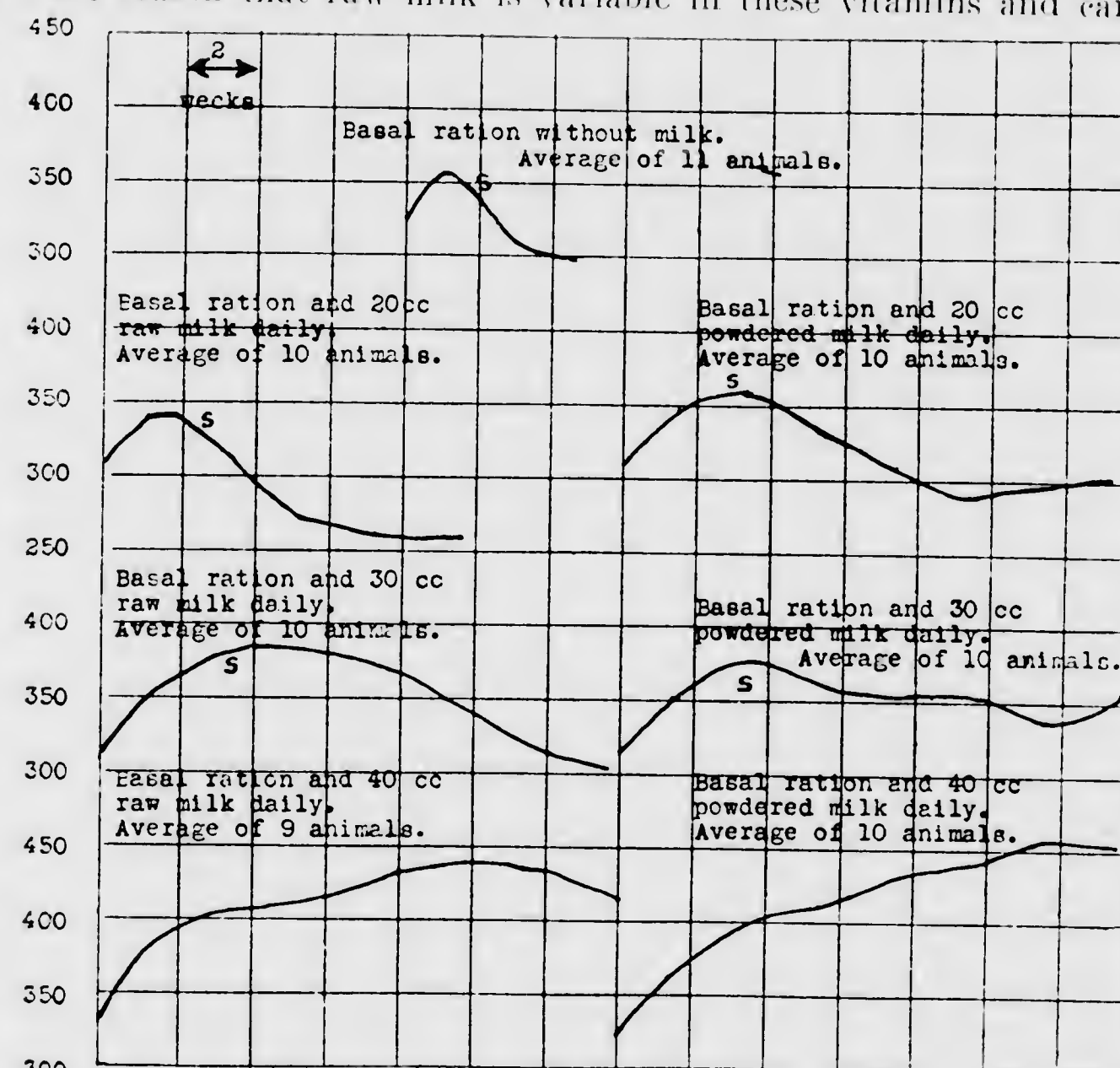


FIG. 6. GROWTH CURVES OF GUINEA PIGS SHOWING THAT IT IS POSSIBLE TO PRODUCE DRIED OR POWDERED MILK WHICH COMPARES FAVORABLY, IN ITS CONTENT OF VITAMIN C, WITH THE RAW MILK FROM WHICH IT WAS MADE.

(Data of Cavanaugh, Dutcher, and Hall.) (Courtesy of the Journal of Industrial and Engineering Chemistry.)

scientific supervision is necessary to prevent vitamin destruction due to careless methods of manufacture. Humphrey (66) describes experiments in which orange juice, dried by the spray process and stored under vacuum for five years, was found to be effective in preventing scurvy in guinea pigs. Givens and Maey (67) found that five out of seven dehydrated fruit juices possessed measurable amounts of vitamin C.

Morgan and Field (68) have published the results of carefully controlled experiments in which dried fruits have been prepared in the Fruit Products Laboratory of the University of California. These desiccated fruits were prepared in different ways and some were treated with sulfur dioxide and some were not. It was found that the sulfur dioxide tended to preserve vitamin C to the extent that the dried fruit retained the full antiscorbutic potency of the fresh fruit. This was not true of the "unsulfured" fruit.

A study of vitamin A revealed the fact that different fruits vary in their ability to retain vitamin A during "sulfuring" and dehydration. Peaches retained 86 to 100 per cent of their original vitamin A content, while prunes varied from 24 to 91 per cent. Apricots, which were richer in vitamin A than the fruits just mentioned, varied from 16 to 51 per cent in the amount of vitamin A retained. These workers postulate differences in the type and amount of oxidative catalysts present in the different fruits. It is interesting to note that they found that yellow peaches and apricots compared favorably with spinach, eggs and butter with regard to their content of vitamin A.

(2) Pasteurization and Evaporation

As early as 1894 Barlow (69) stated that "cooked milk" was inferior to raw milk in antiscorbutic potency. It is possible to find many statements by physicians to the effect that pasteurization and heating should be avoided. Lane-Clayton (70), however, has marshalled a most impressive array of research and clinical data to show that the nutritive value of milk is not harmed by heat treatment.

In 1914, Hess and Fish (71) pointed out that the antiscorbutic value of milk was slowly destroyed by heating but that intense heat for a short time was less destructive than a lower temperature for a longer period. Barnes and Hume (72) reported in 1919 that about one half of the antiscorbutic potency of milk was destroyed by boiling the milk over a gas flame. In the same year Hart, Steenbock and Smith (73) stated that they obtained destruction by heating milk at 120° C. for ten minutes.

The discovery by Zilva in 1920 (74) that vitamin A could be destroyed by ozone was followed by the almost simultaneous announcements of Hopkins (75) and of Drummond and Coward (76) that atmospheric oxygen possessed destructive properties.

In 1921 Miss Edla Anderson (77), working in the writer's laboratory, conducted experiments which showed quite conclusively that milk could be pasteurized for 30 minutes at 63° C. without appreciable destruction of vitamin C. If, however, air, oxygen or hydrogen peroxide were introduced, the vitamin was destroyed completely. During the same year Harshaw and Hall (78), working in the same laboratory, reported similar observations on orange juice. It was possible to destroy the antiscorbutic factor in orange juice with oxygen and hydrogen peroxide at room temperature, although the speed of destruction, like other chemical reactions, was enhanced by heating.

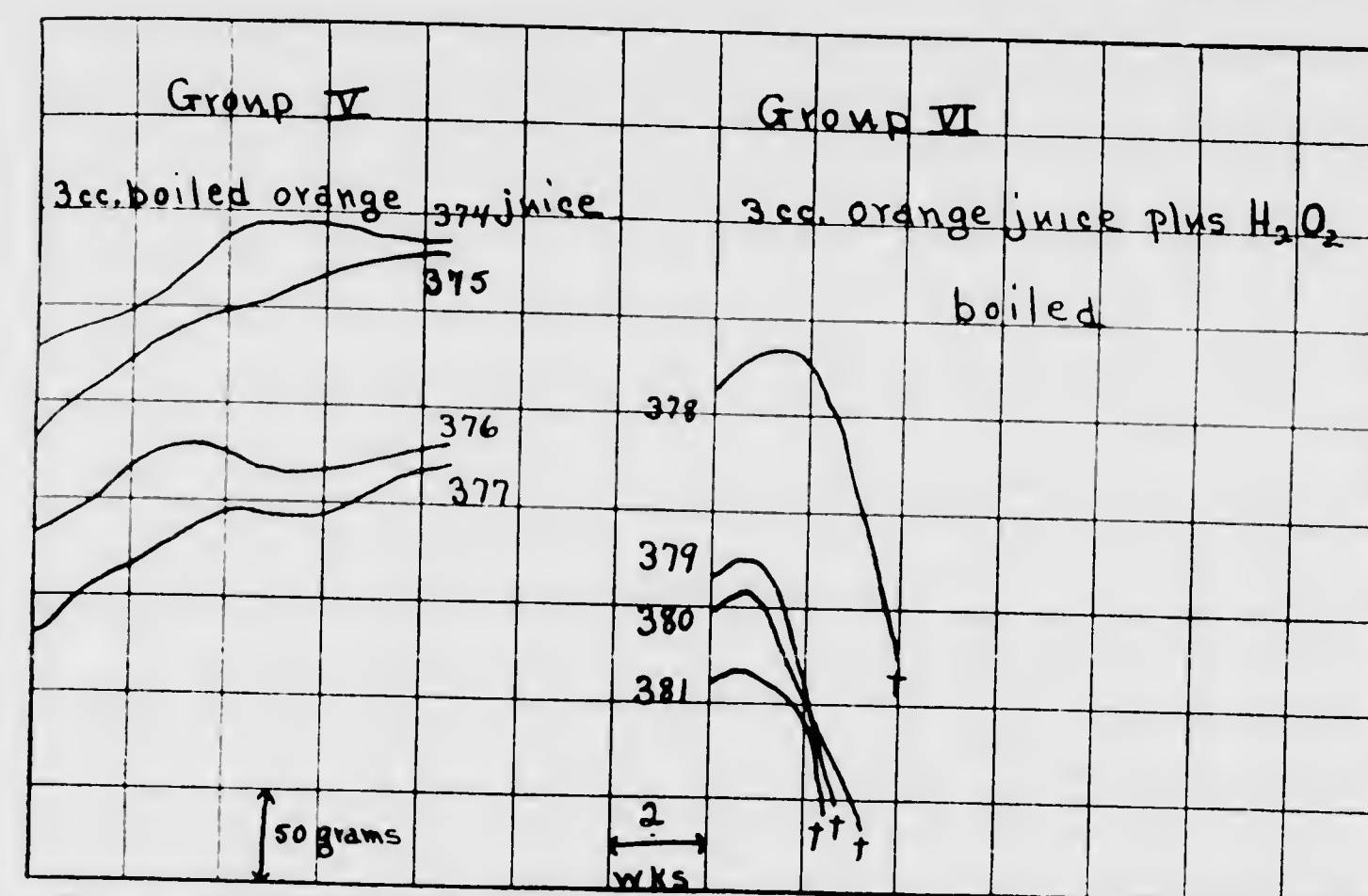


FIG. 7. SHOWING THE GROWTH CURVES OF GUINEA PIGS WHICH WERE FED A SCURVY-PRODUCING DIET.

Group 5 received 3 cc. of boiled orange juice (daily) and grew quite well and showed no evidence of scurvy. Group 6 received 3 cc. of boiled orange juice (daily) to which had been added a small quantity of hydrogen peroxide, which is an oxidizing reagent. All guinea pigs in group 6 died in four weeks or less and showed marked evidence of scurvy. (Data of Dutcher, Harshaw and Hall.) (Courtesy of the Journal of Biological Chemistry.)

In 1926 Dutcher, Francis and Combs (79) published the results of a three-year study on the effect of commercial evaporation on the vitamin B complex in milk. The evaporated milks were made in the Department of Dairy Husbandry in commercial evaporators, which were of two types, viz. (a) Vacuum pan, and (b) air blast evaporator. The evaporated milks were reconstituted with distilled water and fed side by side with equivalent amounts of the raw milk from which the evaporated milks were made. We were unable to detect appreciable destruction of the vitamin B complex.

A subsequent study of the stability of vitamin A (80) led us to conclude that some destruction of vitamin A had occurred, that this destruction was increased by aeration and sterilization, but that the destructive effect was not of sufficient magnitude (10 to 30 per cent) to cause concern.

A similar study (81) led us to conclude that the ossifying potency of evaporated milk is lowered during the evaporating process. We were unable to state how much of this was due to mineral losses during evaporation and how much was due to destruction of vitamin D. Since milk, at best, cannot be considered a rich source of vitamin D, this loss of ossifying potency does not assume serious proportions.

Since the vacuum process of evaporation does not necessarily cause serious vitamin destruction, it is to be expected that concentrated orange

juice, tomato juice and other fruit juices can be made without serious destruction of vitamin C (82).

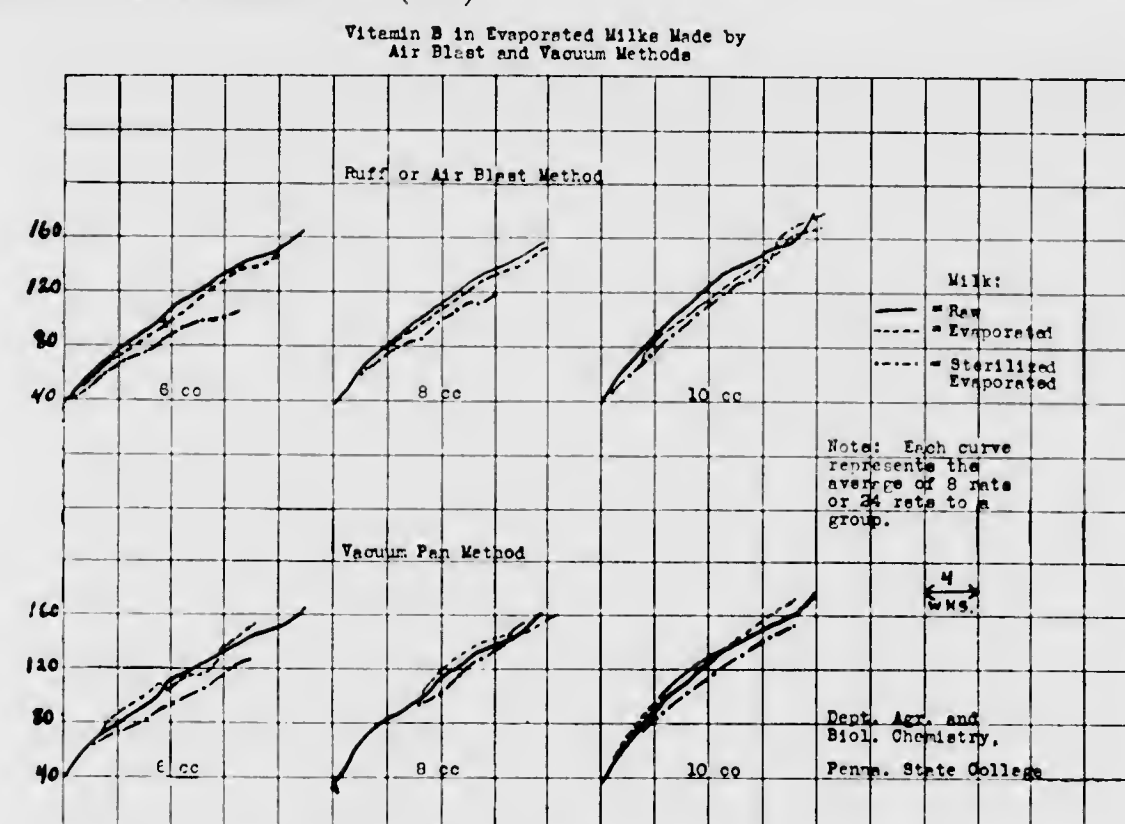


FIG. 8. GROWTH CURVES REPRESENTING GROUPS OF RATS WHICH RECEIVED A BASAL RATION DEFICIENT IN THE VITAMIN B COMPLEX.

The data indicate that the evaporation process and subsequent sterilization do not cause sufficient destruction of the Vitamin B complex to cause concern. (Data of Dutcher, Francis, and Combs.) (Courtesy of the Journal of Dairy Science.)

(3) Cooking and Canning

Holst and Frølich, of Christiania, Norway, were the first (83) to prove, in a practical way, that experimental scurvy would be produced in guinea pigs by feeding foods identical with or similar to those which were known to cause ship-beriberi and ship-scurvy in humans. It was during this epoch-making series of investigations (1907-1912) that they found that they were able to prevent or cure the disease by feeding a number of fresh fruits and vegetables.

In subsequent work (84) these resourceful investigators made many observations and discoveries, regarding the stability of what later became known as vitamin C, that have stood the test of time and the subsequent refinement of experimental methods of feeding. For example, they studied the effect of temperature and time of cooking of cabbage and found that continuous cooking was deleterious. They discovered the stabilizing influence of acids and the destructive effect of alkalies. And, what was most remarkable and significant, these keen observers noted that the destructive effect of cooking seemed to be less marked if air was excluded during the cooking. They also noted the destruction of antiscorbutic properties when vegetables were dried and recorded the fact that the residual scurvy-curing properties could be preserved best by keeping the desiccated materials absolutely dry, rather than to store them where they could gradually take up atmospheric moisture.

Since that time additional information of great value has been gained, to be sure, but most of the work has served to amplify and refine the first fundamental observations of these pioneer workers relative to the wisdom of quick drying, short periods of cooking, elimination of oxygen and the importance of hydrogen-ion concentration.

Of the more recent work, that of Kohman and Eddy and coworkers seems to be of the greatest interest from a practical standpoint. For nearly ten years these investigators have studied the various phases of heating, cooking and sterilization which might lead to more intelligent and scientific methods of processing canned fruits and vegetables in order that they might retain a maximum amount of the original vitamins contained in the raw materials (85). This has been accomplished by removing dissolved oxygen, in water and in food materials, by vacuum and by steam.

In the case of apples, it was found that vitamin C preservation could be accomplished by pre-treating with dilute brine to allow cellular respiration to use up the available tissue oxygen, after which the apples are processed in the absence of air with no loss of vitamin C. They noted a loss of vitamin C when raw apples were stored from October to March but could detect no loss of vitamin C in canned apples after eight months of storage. While cold packed tomatoes were practically identical in vitamin potency with the raw tomatoes from which they were made, open kettle tomatoes seemed to have lost some of their antiscorbutic potency.

Vitamin A was found to be practically unaffected by commercial canning methods but was lost in the pulp if the tomatoes were filtered. Vitamin B was partially destroyed and was shown to be susceptible to oxidation. Recent work has shown that tomato stock can be concentrated to two-fifths the original volume by open kettle boiling without appreciable destruction of vitamin C, using a blanket of steam to avoid contact with atmospheric oxygen.

These investigators have succeeded in rearing several generations of rats and guinea pigs on diets consisting solely of canned foods, showing that modern scientific methods of manufacture have progressed to the point where canned goods are practically equal, in nutritive value, to the fresh food materials from which they were made.

It is evident that oxidation and over-cooking should be avoided so far as vitamins A and C are concerned. This has been emphasized by many workers, especially by Chick and Dalyell (86), who showed in a most practical manner that much of the scurvy in children in a Vienna Hospital was due to over-cooking the food.

(d) Irradiation

This phase of food treatment is still in the experimental stage (commercially) and its importance and significance remain to be ascertained. A number of food manufacturers have obtained licenses from the Wisconsin Alumni Research Foundation to irradiate their products with

ultra violet light to endow them with antirachitic potency. It is true that most of our common foods are deficient in vitamin D and it is generally recognized that growing children can ingest antirachitic foods with profit during the winter months, when the children are less likely to be exposed to the beneficial action of sunlight.

The discovery that the alcohol, ergosterol, can be activated by ultra violet light with the formation of vitamin D has led to the commercial production of Viosterol. This product can be made 100,000 to 150,000 times more potent than a standard cod liver oil. Viosterol has found a wide use by physicians in the treatment of rickets although it cannot be considered a substitute for cod liver oil, since it does not contain vitamin A. Viosterol or irradiated ergosterol may be added to commercial food preparations where it is desirable to introduce vitamin D without actually irradiating the food. This is being done at the present time with certain human foods and with certain types of poultry feeds.

In our enthusiasm for and our interest in vitamins and deficiency diseases we should try to preserve a scientific and sensible attitude toward the whole question of vitamin therapy and diet construction. Let us recognize and appreciate the importance of vitamins, but in our anxiety to include them in our dietary we must not allow ourselves to condemn and discard many valuable foodstuffs because they happen to be vitamin deficient.

There has been a tendency on the part of some workers to condemn white bread, white rice, and other foods because of their lack of certain of these food factors. Such foods have a definite economic and nutritional place in the normal diet and must be supplemented by vitamin-containing foods but it is not wise or necessary to condemn them or to omit them from the diet.

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The Effect of Irradiation

ON THE

Electrophoretic Velocity, Viability, Agglutinability, Lysis, and pH Of Escherichia Coli

[Technical Bulletin]

THE PENNSYLVANIA STATE COLLEGE
SCHOOL OF AGRICULTURE AND EXPERIMENT STATION
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The Effect of Irradiation on the Electrophoretic Velocity, Agglutinability, Lysis, Viability, and pH of *Escherichia Coli*^{1, 2}

BY MARTIN W. LISSE AND RALPH P. TITSLER, WITH THE TECHNICAL ASSISTANCE OF GEORGE R. SHARPLESS

PART ONE

Introduction

THE STUDY of photochemistry has been highly stimulated through the study of ultraviolet radiation, and much knowledge concerning the effect of these rays on inorganic and organic substances, on both dead material and living cells and tissues, has been obtained. This knowledge has been summarized in books, such as Ellis and Wells (1925), Luckiesh and Pacini (1926), Mayer (1926), and Morton (1928). Excellent bibliographies are given by Mayer (1926), Bersa (1927) and Laurens (1928).

Most of the work relative to the effect of irradiation of bacteria has reference to sterilization and concerns itself with the bactericidal and stimulative effects of rays of the different wave lengths. There has been some theoretical discussion, but no satisfactory answer has been given as yet, concerning the mechanism by which radiation, and especially ultraviolet radiation, exerts its action on cells and tissues. Any experiments, therefore, which will throw some light on this mechanism, which is probably very complex, are likely to be productive of significant results, especially in the fields of disinfection, biology, and medicine.

An application of the principles of colloid chemistry has led us to suspect that irradiation of bacteria with ultraviolet rays is accompanied by changes in the electrical charge of bacteria (measurable electrophoretically), and that many of the properties of bacteria are a function of this charge or capable of being evaluated by it. Mayer, p. 355 (1926), and Norton in Jordan and Falk, p. 377 (1928), have made similar hypotheses.

Review of Literature

A. THE EFFECTIVE WAVE LENGTHS

That by far the most intensive bactericidal action of light is concentrated in the ultraviolet region one learns by a review of papers

¹This is a resumé of a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agricultural and Biological Chemistry in the Graduate School of The Pennsylvania State College, 1929.

²For preliminary paper see Proc. of the Soc. for Exp. Biol. and Med., 28, 311, 1931.

by Downes and Blunt (1877), Ward and Franklin (1892 and 1893), Bie and Bang [pp. 238-239, Ellis and Wells (1925)], Barnard and Morgan (1903), Hertel (1905), Thiele and Wolf (1906), Cernovodeanu and Henri (1910), Bazzoni (1914), De Voogt (1916), Newcomer (1917), Bayne-Jones and Van der Lingen (1923), Coblenz and Fulton (1924), Burger (1928), and Gates (1929b and 1930).

Coblenz and Fulton (1924) even found how many watts of energy per bacterium were necessary to kill.

Gates (1929a) showed that very different total incident energies were required at different wave lengths to produce similar effects in bactericidal action.

Though one does not make the Schumann region available in ordinary practice, several investigations have dealt with its effect on bacteria (Lyman, 1910; Bovie, 1915-1916).

Fulton and Coblenz (1929) found that the germicidal action for *Bacillus coli* was produced by rays extending from the shortest wave length tested, 2,400 Å. U., to and including 3,650 Å. U., and that the action probably extends beyond this upper limit, but to a very weak degree.

B. THE INFLUENCE OF VARIOUS FACTORS

a. Bacterial Medium

See papers by Kreusler (1901), Barnard and Morgan (1903), Lyman (1910), Young and Pingree (1913), Bayne-Jones and Van der Lingen (1923), Coblenz and Fulton (1924), Hill (1924), and Gates (1929b).

b. Time and Temperature of Irradiation

The temperature coefficient should be low as Bayne-Jones and Van der Lingen (1923) reported. Since photochemical reactions in general are nearly independent of temperature changes, one might suspect that the speed of the reaction might not be greatly affected by such changes, in so far as the reaction (change of charge, killing, etc.) is merely a photochemical one. Gates (1929b) stated that since the temperature coefficient of the bactericidal reaction approaches 1, evidence is furnished for the belief that the direct action of ultraviolet light on bacteria is essentially physical or photochemical in character. These values are opposed to those by Thiele and Wolf (1907) and by Wiesner (1907) but in agreement with the report of Cernovodeanu and Henri (1910).

See papers by Bovie and Klein (1919) and Bovie and Daland (1923) for heat sensitization studies and by Bovie (1915-1916), Coblenz and Fulton (1924), and Norton, pp. 374-377 in Jordan and Falk (1928), for studies on intermittent exposure.

c. Bacterial Species

See Cernovodeanu and Henri (1910) and Burge and Neill (1915).

d. *Relative Intensity*

It is well known [Pacini (1923), Harrison and Forbes (1925), Luckiesh and Pacini, p. 211 (1926) and Gates (loc. cit.)] that the relative intensities of the various radiations or spectral lines of ultraviolet rays vary with such factors as current density, voltage, etc.

Coblentz and Fulton (1924) showed that for low intensities of ultraviolet radiation a stimulation of bacterial growth may possibly occur, whereas for high intensities a bactericidal effect is obtained.

e. *Source of Ultraviolet*

Patton (1926) reported that a water-cooled quartz mercury lamp is far more desirable for bactericidal action than an air-cooled one. A National Carbon Company pamphlet (references) describes carbons A to K, and states that carbons of type B or K give a spectrum more like a water-cooled quartz mercury lamp than the other types, and that the rays given from them are more highly bactericidal than those from other carbons.

f. *Dosage of Irradiation*

O'Donnell (1927) showed that small doses of ultraviolet rays can increase irritability whereas large doses can act as depressants, and that the irritability is not due to increased permeability.

g. *Counteracting Properties*

Pech (1920, 1925), Le Bon (1920), and Benoit and Helbronner (1923) suggested that there are numerous cases in which the physicochemical reactions are affected in different manners by different radiations, thus causing a counteracting effect. That the longer wave lengths of ultraviolet are stimulative to development whereas the shorter ones are lethal was shown by Higgins and Sheard (1926). Hinrichs (1928) stated that the amount of radiation in a given region of the spectrum, which is absorbed by the protoplasm in a given period of time, determines whether there will be an increase or decrease of the physiological processes.

C. EFFECT OF ULTRAVIOLET RAYS ON VARIOUS REACTIONS AND SUBSTANCES

Bovie (1913) showed that irradiation with ultraviolet coagulates proteins. The toxic action of light on protoplasm probably results from the absorption of light by aromatic amino acids which are constituents of cell protein [Houghton and Davis (1914), Harris and Hoyt (1917, 1919) and Pincussen (1924)]. Gates (1928) reported that nuclear derivatives are also probably responsible for the absorption of ultraviolet radiation. Bovie (1913) showed that light coagulation, like heat coagulation of proteins, involves two reactions of different temperature coefficients. Pincussen (1924) reported that colloids generally tend

toward greater aggregation under ultraviolet light. Mischenko (1927) stated that proteins, of low molecular weight only, undergo cleavage on irradiation. Spiegel-Adolf (1929) made a number of physicochemical studies on irradiated proteins. Janet Clark (1922) stated that the quantity of charge carried by protein particles can be altered by exposures to ultraviolet light, but that precipitated colloid retains its characteristic chemical properties. Koertz (1923) suggested that irradiation affects cell protein rather than surface colloids. Mond (1922, 1923) noted a difference in the effect of ultraviolet irradiation of albumin solutions versus globulin and fibrinogen solutions. Abderhalden and Rossner (1928) gave ultraviolet absorption curves of alpha amino acids, polypeptides, etc. Marchlewski and Wierzuchowska (1928) did the same for proteins.

Working with egg albumin suspensions, Clark (1922) concluded that irradiation produces a state of greater aggregation when the albumin particles are negatively charged and one of greater dispersion when they are uncharged or charged positively. When the pH is less than 4.8 (isoelectric point) the supposed escape of electrons (photoelectric effect) following irradiation increases the quantity of the positive electrical charge. Negatively charged particles lose part or all of their charge and may become positively charged. Mayer, p. 352 (1926), states that irradiation of proteins may cause changes opposite to coagulation.

MacCallum (1916) states that cell lipoids are sensitized by ultraviolet rays and are thus prepared for enzymatic decomposition. Because of greater intensity of this enzyme action, tissues may be destroyed by autolysis or lysis. Schwarz (1903) thought that light action may be largely due to liberation of lecithin. Schulz (1910) and Hoffman (1919) attributed toxic action of light to photoproducts of the lecithins.

There are some authors who believe that the effects of ultraviolet light on cells and tissues are explained by its action on cellular enzymes [Bering and Meyer (1912), Neuberg (1914), and Pincussen (1923)]. Agulhon (1911) divides enzymes into three classes on the basis of the effect of radiations upon them. There are numerous articles to show that ultraviolet light inactivates enzymes [Ziber (1913), Burge, Fisher and Neill (1916), Svanberg (1921), Pincussen et al. (1923-1929), Calvin (1930), Gorbach and Lereh (1930), and Morgulis (1930)]. No influence on the catalytic activity of enzymes was reported by Kawakami (1929) and by Takamuja (1929).

D. MECHANISM OF BACTERICIDAL ACTION OF LIGHT

In accordance with the laws of photochemistry, bactericidal rays must be those that are absorbed by the material acted upon. When ultraviolet rays were passed through aqueous bacterial suspensions, and the spectrum of the emergent rays photographed, Browning and Russ (1917) and Bayne-Jones and Van der Lingen (1923) found absorption

bands in the region of the rays which killed the bacteria. Henri (1913) showed that rays which have the maximum bactericidal power are absorbed by proteins.

In experiments in which solutions of tyrosin and aminobenzoic acid in quartz beakers were interposed between the source of the ultraviolet rays and the bacteria, Harris and Hoyt (1917, 1919) stated that bacteria were protected for long times. Houghton and Davis (1914) reported that aromatic and not aliphatic amino acids are the protective agents.

Some authors have thought that the nucleus of bacteria appears to be more absorptive than the remaining portion of the cell, and that the nuclei are attacked and thus the intracellular enzymes are destroyed, a process leading to death of the organism. This theory is not generally accepted, according to Burge (1916, 1917). It is true, however, that the nuclei contain substances which are especially sensitive to ultraviolet rays (such as phenyl-alanin and tyrosin) to a much greater extent than other tissues [Gates (1928)], and perhaps it is the pronounced presence of these two substances which makes a bacterial cell more responsive to radiation than a normal tissue cell (p. 212, Luckiesh and Pacini, 1926).

The tendency has been to say that the lethal effect of ultraviolet rays is not due to any germicidal substances such as hydrogen peroxide and ozone, produced in the medium or environment [Courmont (1911), Bujwid (1911), Blom (1913), Harris and Hoyt (1917), Fair (1920)] yet Bedford (1927) maintained that the ultraviolet rays exert an influence on microorganisms by virtue of the hydrogen peroxide which they produce within themselves. Coblentz and Fulton (1924), believing that no toxic chemical substances produced by the action of the rays on the medium are responsible for killing of bacteria, stated that when the media used were given greatly prolonged preexposures, subsequent seeding of *B. coli* on the surface of the preexposed agar gave little or no growth, and that the effect is not due to heat radiation.

In view of the facts already cited, Norton (pp. 374-377, Jordan and Falk, 1928) and Glaser (p. 245, Ellis and Wells, 1925) believe that the lethal mechanism is a direct one. In view of the fact that ultraviolet rays produce changes in both inorganic and organic colloids [Cernovodeanu and Henri (1910), Clark (1925)], which changes are probably accompanied by changes in charge, Norton (loc. cit.) recently suggested that the changes which bacteria suffer when exposed to ultraviolet rays are "accompanied, perhaps preceded by, changes in the electrical charges of the bacteria." This hypothesis was also made by us.

Of very special interest are a number of papers reported after or about the time this work was started, all of which helped to strengthen the hypothesis just stated. Beaver and Muller (1928) stated that some red gold sols change to blue on exposure to ultraviolet rays, but by prolonged irradiation they are peptized to stable red sols. Recent

work on phagocytosis [Falk and Matsuda (1926), Ponder (1926)], Abramson (1927), Mudd et al. (1928), Lucke et al. (1929)] makes it apparent that the organisms which are more readily and more extensively phagocytized have the lower potential difference. Falk and Reed (1926), working on the alterations in cell electrophoretic potential produced by direct irradiation of blood in vivo, showed a slight decrease of potential difference.

Of very special interest also are the numerous papers recently published on the relationship between potential difference, charge, virulence, etc. [Winslow, Falk and Caulfield (1923), Falk and coworkers, and Jensen and coworkers (1925-1930), Shaughnessy and Crisswell (1925), Tittsler and Lisse (1928), Zucker (1929), Rane (1929)].

Prior to these reports there have been several minor reports of such a parallelism [Girard and Audubert (1918), Shearer (1919-1922), Northrop and DeKruif (1922)]. Experiments with bacteria, pathogenic for plants, gave similar parallelisms [Sharp; Link and Sharp; Falk, Sharp and Link (all 1927), and Link (1928)]. For relationships between "Smooth" and "Rough" strains and between virulence and potential difference see Hadley (1927, 1928), Jacobson and Falk, Link and Hull, Sharp (all 1927), and Kahn and Schwarzkopf (1931).

In view of the relationship between virulence and potential difference, one might ask by what constituents or properties of the cell the potential difference is determined. The answer might lie with permeability phenomena, if the origin of the potential difference is explained on the basis of the Donnan equilibrium [Winslow, Falk and Caulfield (1923)], or with the effect of specific soluble substances (toxins, etc.) on the cell's charge [Cole (1917), Felton and Bailey (1926), Sia (1926), Heidelberger (1927)], or a combination of the two [Jensen (1926, 1927), Falk, p. 573, Jordan and Falk (1928)].

Since the Donnan equilibrium is thought by some to be related to the electrophoretic potential, and since many colloid chemists think that such potentials and thermodynamic potentials are not the same thing (see for instance Kruyt, 1927), it might be well to quote from a summary of a paper by Loeb (1923) on membrane potentials and electrophoretic potentials: "The forces inherent in the protein particles and linked with the membrane equilibrium prevail to such an extent over the forces inherent in the water, that the sense of the cataphoretic migration of protein particles is determined by the forces resulting from the membrane equilibrium." (See also Loeb, 1924).

In the Mayo Foundation Lectures, Millikan (1927) states that the photoelectric effect is important because it furnishes the most simple and convenient way by which charges on colloid particles can be changed and because the therapeutic effects of ultraviolet rays are necessarily fundamentally attributed to the fact that they have the power of detaching electrons from the surface.

Clark (1922) proposed a general theory of light action as it affects

changes in protoplasm, according to which physiological actions of radiations are due to photoelectric action of light, which by producing an ionized condition in light-sensitive molecules, leads to subsequent chemical reactions. Bovie (1915, 1916, 1918, 1920, 1925) [see also Mayer (1921), Bovie and Daland (1923), p. 358, Mayer (1926)] further elaborates the *modus operandi* of the essential principles of this theory. He states that protoplasm is a polyphase colloidal system whose interfaces are molecularly and electrically organized and oriented, and that this organization is destroyed by death, that the action of light on biochemical reactions involves the basic phenomena of life, and that these are colloidal and electrical phenomena. The explanations of Clark and Bovie have been looked upon as the best yet offered.

Bovie (1913) noted that if the decomposition of the protein molecule and the disorganization of the protoplasm is not carried too far, there is a stimulation of the cell, but that this stimulation, if not followed by recovery, is followed by lysis.

Though it would take us too far afield to make a complete review of Lillie's chapter in Cowdry's *Cytology* (1924) entitled "Reactivity of Cell," suffice it to say that he calls attention to the fact that all reactivity of cells, be it stimulation, recovery, or response, is accompanied by variations in the electrical potential and by changes in permeability. The electrical potential referred to is, however, of the membrane type and perhaps due to a Donnan equilibrium.

As a result of studies of the temperature coefficient of the effect of light on bacteria by Bayne-Jones and Van der Lingen (1923), Higgins and Sheard (1926), Dognan and Tsang (1928), and Gates (1929b), it does not appear impossible that the primary effect of ultraviolet irradiation is structural in nature, being a change in permeability of cell membrane, or a change in the colloidal substratum and surface layer of protoplasm from which specific chemical effects follow. One must not forget, however, that most photochemical processes have small temperature coefficients, and thus possibly draw erroneous conclusions (from a study of temperature coefficients) relative to that reaction which controls the velocities of a complex series of reactions.

Still other viewpoints on the mechanism of the action of ultraviolet rays on cells are those of Noack (1920) and Tehahotine (1921).

In concluding the discussion of the mode of action of ultraviolet light, we will quote from (p. 74) Laurens (1928): "It is evident, while we are in possession of an enormous amount of information concerning the results of irradiating man and animals, normal and abnormal, we are not yet ready for explanations and generalizations, and no unitary hypothesis as to how the results described are obtained can yet be formulated. We still stand in need of facts obtained under definite, controlled conditions of dosage, intensity, wave length, etc., on normal and abnormal organisms, and when sufficient quantitative data have been thus obtained a theory will develop as a matter of course."

E. ELECTROPHORETIC AND AGGLUTINATION STUDIES WITH BACTERIA

For a comprehensive review of electrophoretic studies with bacteria and of bacterial agglutination see Tittsler and Lisse (1928).

F. LYSIS

Bovie and Hughes (1918, 1919) in studies of cytolysis caused by radiation state that the percentage of cytolysis increased with length of exposure, and that it occurs when a certain amount of a toxic photo-product is formed.

The Problem

Knowing that radiation kills bacteria and that the ultraviolet portion of the spectrum is the more active portion, especially in its shorter wave lengths, and reasoning that the radiation, when absorbed, could cause some change in the protein and other colloids of the bacterium, probably a coagulation which would affect the permeability of its membrane and therefore the Donnan equilibrium and the ionic environment, we came to the conclusion that a change in the electrokinetic potential of the organisms would accompany the process. Reasoning according to the theoretical views held by Clark and by Bovie, and knowing that most organisms, *Escherichia coli* included, are negatively charged, we again arrived at the conclusion that the irradiation would be accompanied by a decrease of negative potential.

Knowing that ultraviolet radiations possess an inactivating and bactericidal power and having previously suggested (as have others) that electrokinetic potential, agglutinability and virulence are related, the suggestion again presented itself that the electrokinetic potential would be affected by such radiation. Our previous work suggested that decreases in potential difference should parallel abilities to be agglutinated.

There was enough written historically to make us ready to accept an initial stimulative effect if it should be found, and to look for a change in the ionic concentration of the bacterial medium.

This part of the paper, therefore, attempts to show that the ultraviolet irradiation of aqueous suspensions of washed living *Esch. coli*, except on very brief irradiation, brings about a decrease in electrophoretic potential (charge) which is paralleled by greater agglutinability and that any explanation of the effect of irradiation must take into consideration a mechanism which can account for this change of charge. Lysis and bactericidal effects which accompanied irradiation were studied.

Since there was a possibility of a change of electrophoretic velocity being produced during storage of rayed and unrayed samples, or during exposure of samples to the atmosphere of the laboratory in which the irradiation took place, experiments were performed to demonstrate that any change observed was not due to these factors.

Technique

A. PREPARATION OF SUSPENSION

Cultures of *Esch. coli*, grown on proteose-peptone agar, were suspended in distilled water of pH 6.0 to 6.3, essentially by the technique reported by Tittsler and Lisse (1928). These suspensions were not heat-killed.

B. IRRADIATION

Using a carbon-arc lamp, supplied with "Therapeutic B" carbon electrodes in order to obtain a high proportion of bactericidal rays, and operating at approximately 90 volts and 3 amperes, 25cc. of the bacterial suspensions approximately 3 mm. deep, in large Petri dishes (with covers off), at approximately 30°C., were irradiated at 35.6 cm. from the electrodes at intervals of time varying from 2 to 60 minutes. At the same time other samples were exposed to the laboratory environment in a similar manner for similar times. After such treatment the suspensions, made up to the original volume with distilled water in order that comparative figures could be obtained in all tests made, were placed in rubber-stoppered flasks. These samples as well as the unirradiated and exposed samples were now ready for the electrophoresis studies, which were never made later than ten hours after treatment, and usually very much sooner.

The lamp was permitted to operate for five minutes prior to irradiation thus assuring constant light intensity and quality. All glassware was cleaned with the utmost care. Some of the irradiation was done through Corex A glass, which was later omitted because it delayed the effect of the irradiation.

C. STORAGE AND EXPOSURE

Electrophoretic measurements were made on samples of the aqueous bacterial suspensions of non-irradiated bacteria after all the other measurements of the irradiated samples were made, and also on suspensions which stood exposed in Petri dishes near to the carbon-arc lamp during the time of irradiation but which did not receive the radiation. The former were called "stored" and the latter "exposed." These measurements served to show that storage and exposure to the atmosphere of the laboratory during irradiation were not the causes of any changes in electrophoretic velocity which were observed.

D. MEASUREMENT OF ELECTROPHORETIC VELOCITY

The electrophoretic measurements were made in a Northrop Kunitz cell as described by Tittsler and Lisse (1928) with these exceptions: (a) No salt solution was used in the final washing of the cell since aqueous suspensions were used. (b) The migration velocities of bacteria were read across a distance of 300 micra under direct current

of approximately 90 volts and a potential gradient of approximately 3. (c) Readings were made at depths 1/6, 5/12, 1/2, and 7/12, 20 to 30 readings being taken at depths 1/6 and 1/2 and enough (usually 10) at 5/12 to 7/12 to ascertain that the proper depths were actually attained, known by the fact that readings at depth 1/2 showed a maximum in accordance with the Smoluchowski curve (1921). The maximum velocities are relative values and give the sum of electrophoretic velocities and those due to return-flow of water previously put in motion by electroendosmose.

Actual electrophoretic velocities were obtained according to the formula:

$$V = 3/4 v' \text{ at } 1/6 + 1/4 v' \text{ at } 1/2,$$

V and v' being actual and observed velocities respectively. Both maximum (relative) and actual velocities are calculated in $\frac{\mu}{\text{sec.}}$

per $\frac{\text{volt}}{\text{cm.}}$ as averaged averages, which means that the readings made

at each depth were averaged and then used to calculate an average value for actual and maximum values of migration velocities for each sample prepared. If the electrophoretic work on any sample was repeated, such average values were again obtained. All such averages were averaged to get what is called an averaged average in the work reported. If the final value reported is one resulting from twelve averaged averages it means the twelve samples were prepared at twelve different times from twelve different sets of slants, and an averaged average obtained in each case, the average of these giving the final value.

E. AGGLUTINATION STUDIES

These studies were made practically as described by Tittsler and Lisse (1928). Two antisera, preserved by glycerine, and of equal agglutinating ability were used, and the results averaged. The bacterial suspensions were mixed with the antisera in equal parts at the different dilutions. 1.7 per cent sodium chloride was used in making the serum dilutions so that a concentration of .85 per cent would be present in the final dilutions. The tubes were read at definite intervals after 24 to 66 hours incubation at 37° C., "5" indicating complete flocculation and clearing, such that the medium had become water clear, and "1/2" the slightest agglutination recordable.

F. LYSIS

To determine the lysing effect of irradiation direct counts were made using the method generally employed for the enumeration of blood corpuscles (Kolmer, 1925, p. 194). Average results of duplicate counts of 25 one four-hundredth sq. mm. areas are reported in terms of bacteria per cc. of suspension used and in per cent decrease.

G. BACTERICIDAL STUDIES

To show that irradiation of the bacteria was or was not accompanied by death, duplicate platings were made immediately before and after irradiation. The usual bacteriological procedure of making quantitative plate counts was followed. Nutrient agar was used and the colonies were counted after 24 hours incubation at 37° C. The number of viable bacteria per cc. was calculated.

Experimental Results

A typical set of electrophoresis data, using the Northrop Kunitz cell, is presented in Table I.

TABLE I

Voltage: 90.5		Distance between electrodes 29.9 cm.		Potential gradient: 3.03	
Distance traveled 300u			Time in seconds		
At depth 1/6	21.84	23.28	5.30	5.29	At depth 1/2 or 6/12
	18.58	16.48	5.08	5.00	
	17.22	19.48	4.96	5.41	
	17.54	18.30	5.18	5.29	
	16.20	18.58	5.38	5.41	
	current reversed		current reversed		
	18.98	19.70	5.39	5.11	
	19.74	16.74	5.05	5.33	
	21.28	18.46	5.28	5.25	
	17.24	17.12	5.06	5.12	
17.54	22.10	5.45	5.48		
18.616	Average	19.024	5.213	Average	5.269
	18.82			5.24	
At depth 5/12		5.19	5.67		At depth 7/12
		5.42	5.93		
		5.32	5.50		
		5.21	5.73		
		5.55	6.07		
	current reversed		current reversed		
		5.20	5.67		
		5.26	5.57		
		5.14	5.70		
		5.16	5.73		
	5.20	6.05			
	Average	5.27	5.76		

TABLE II. MIGRATION VELOCITIES (IN MICRA PER SEC. PER VOLT PER CM.). MAXIMUM VALUES (AT DEPTH 1/2) USING COREX SCREEN. SEE GRAPH I.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	12	20.26	
Irradiated 2, 3 or 4 min.	2	17.51	-13.6
Irradiated 5 or 10 min.	3	18.84	-7.0
Irradiated 15 min.	3	17.13	-15.4
Irradiated 30 min.	2	17.71	-12.6
Irradiated 60 min.	2	18.80	-7.2
Non-irradiated and exposed	9	19.69	-2.8
Non-irradiated and stored	6	19.96	-1.5

TABLE III. AS IN TABLE II BUT USING NO SCREEN. SEE GRAPH I.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	12	20.26	
Irradiated 2, 3 or 4 min.	6	21.71	+ 7.2
Irradiated 15 min.	3	15.35	-24.2
Irradiated 30 min.	3	14.78	-27.0
Irradiated 60 min.	4	15.72	-22.4
Non-irradiated and exposed	9	19.69	-2.8
Non-irradiated and stored	6	19.96	-1.5

TABLE IV. MIGRATION VELOCITIES (IN MICRA PER SEC. PER VOLT PER CM.) ACTUAL VALUES ($V=3/4$ V' AT $1/6+1/4$ V' AT $1/2$) USING COREX SCREEN. SEE GRAPH I

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	9	10.99	
Irradiated 2, 3 or 4 min.	2	9.89	-10.0
Irradiated 5 or 10 min.	2	10.14	-7.7
Irradiated 15 min.	3	9.89	-10.0
Irradiated 30 or 60 min.	3	10.24	-6.8
Non-irradiated but exposed	7	11.38	+ 3.5
Non-irradiated but stored	3	10.71	-2.5

TABLE V. AS IN TABLE IV BUT USING NO SCREEN. SEE GRAPH I.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	9	10.99	
Irradiated 2, 3 or 4 min.	3	12.44	+13.2
Irradiated 60 min.	2	8.97	-18.4
Non-irradiated but exposed	7	11.38	+ 3.5
Non-irradiated but stored	3	10.71	-2.5

TABLE VI. AGGLUTINATION vs. RAYING. COREX SCREEN. COOLED. 1/4% FORMALIN ADDED. MADE TO VOLUME.

		Serum Dilutions						
		40	80	160	320	640	1280	2560
Read after 24 hrs.	Non-irradiated	3	1/2	0	0	0	0	0
	Irradiated 2 min.	2	0	0	0	0	0	0
	Irradiated 5 min.	3	0	0	0	0	0	0
	Irradiated 10 min.	2	0	0	0	0	0	0
	Irradiated 15 min.	1	0	0	0	0	0	0
	Irradiated 30 min.	2	0	0	0	0	0	0
	Irradiated 60 min.	1	0	0	0	0	0	0
	Irradiated 60 min.	1	0	0	0	0	0	0
Read after 48 hrs.	Non-irradiated	4	1	1	1	1	1/2	0
	Irradiated 2 min.	4	1/2	1	1/2	0	0	0
	Irradiated 5 min.	4	1	1/2	1/2	1/2	1/2	0
	Irradiated 10 min.	4	1	1	1/2	1/2	1/2	0
	Irradiated 15 min.	3	1	0	1/2	1/2	1/2	0
	Irradiated 30 min.	4	1	0	1/2	1/2	0	0
	Irradiated 60 min.	4	1/2	1/2	1/2	0	0	0
	Irradiated 60 min.	4	1/2	1/2	1/2	0	0	0
Read after 66 hrs.	Non-irradiated	4	1/2	0	0	0	0	0
	Irradiated 2 min.	4	1/2	0	0	0	0	0
	Irradiated 5 min.	4	1/2	0	0	0	0	0
	Irradiated 10 min.	4	1/2	0	0	0	0	0
	Irradiated 15 min.	2	1/2	0	0	0	0	0
	Irradiated 30 min.	3	1/2	0	0	0	0	0
	Irradiated 60 min.	3	1/2	0	0	0	0	0
	Irradiated 60 min.	3	1/2	0	0	0	0	0

TABLE VII. AGGLUTINATION vs. RAYING. NO SCREEN. COOLED. 1/4% FORMALIN ADDED. MADE TO VOLUME.

		Serum Dilutions								
		40	80	160	320	640	1280	2560	5120	10240
Read after 24 hrs.	Non-irradiated	3	1/2	0	0	0	0	0	0	0
	Irradiated 2 min.	3	1	1	0	0	0	0	0	0
	Irradiated 5 min.	4	5	5	5	4	2	1	0	0
	Irradiated 10 min.	5	5	5	5	5	5	5	4	2
	Irradiated 15 min.	5	5	5	5	5	5	5	4	2
	Irradiated 30 min.	5	5	5	5	5	5	4	4	0
	Irradiated 60 min.	5	5	5	5	5	5	4	3	clear no pre- cipitate
	Irradiated 60 min.	5	5	5	5	5	5	4	3	clear no pre- cipitate
Read after 48 hrs.	Non-irradiated	3	1	1	1	1	1/2	0	0	0
	Irradiated 2 min.	4	1	1	1	1/2	1/2	0	0	0
	Irradiated 5 min.	5	5	5	5	5	4	3	2	0
	Irradiated 10 min.	5	5	5	5	5	5	5	5	4
	Irradiated 15 min.	5	5	5	5	5	5	5	5	3
	Irradiated 30 min.	5	5	5	5	5	5	5	4	0
	Irradiated 60 min.	5	5	5	5	5	5	5	0	0
	Irradiated 60 min.	5	5	5	5	5	5	5	0	0
Read after 66 hrs.	Non-irradiated	4	1/2	0	0	0	0	0	0	0
	Irradiated 2 min.	5	4	3	0	0	0	0	0	0
	Irradiated 3 1/2 min.	5	5	5	5	5	4	2	1	0
	Irradiated 5 min.	5	5	5	5	5	4	3	2	1/2

TABLE VIII. AGGLUTINATION vs. EXPOSURE AT ROOM TEMPERATURE TO ATMOSPHERE OF ROOM IN WHICH ULTRAVIOLET LAMP IS RUNNING, BUT NOT EXPOSED TO ULTRAVIOLET RAYS. 1/4% FORMALIN ADDED.

		Serum Dilutions							
		40	80	160	320	640	1280	2560	5120
Read after 24 hrs.	Non-irradiated	—	0	0	0	0	0	0	0
	Non-irradiated but exposed 80 min.	—	0	0	0	0	0	0	0
	Non-irradiated but exposed 120 min.	—	1/2	0	0	0	0	0	0
Read after 34 hrs.	Non-irradiated	—	1	1	1	1	1/2	0	0
	Non-irradiated but exposed 80 min.	—	1/2	1/2	1/2	1	1	1/2	0
	Non-irradiated but exposed 120 min.	—	1/2	1/2	0	0	0	0	0
Read after 48 hrs.	Non-irradiated	3	1/2	0	0	0	0	0	0
	Non-irradiated but exposed 100 min.	1	0	0	0	0	0	0	0
Read after 66 hrs.	Non-irradiated	4	1/2	0	0	0	0	0	0
	Non-irradiated but exposed 100 min.	2	0	0	0	0	0	0	0

TABLE IX*. SHOWING RELATION BETWEEN LENGTH OF IRRADIATION AND EXTENT OF LYSIS.

Treatment	Count per 1/400 sq. mm.			Bacteria per cc. of suspension used	Per cent decrease
	1st	2nd	Av.		
Non-irradiated	4.03	3.97	4.00	320,000,000	-----
Irradiated 5 min.	3.91	3.91	3.91	312,800,000	2.3
Irradiated 15 min.	3.23	3.34	3.28	262,400,000	18.0
Irradiated 30 min.	2.55	2.41	2.47	197,600,000	38.3
Irradiated 60 min.	1.47	1.42	1.44	115,200,000	64.0

No table is given for bactericidal studies in this series, since two minute irradiation was the shortest irradiation period used and all plates of irradiated samples were sterile, whereas they initially contained 840—1,320 million bacteria.

Discussion of Results

A. TECHNIQUE

It will be observed in Table I that the data presented conform to the Smoluchowski curve. Data that did not thus conform, when the Northrop Kunitz cell was used, were rejected. According to Smoluchowski the velocities at the middle depth (6/12) should be faster than those on either side (5/12 and 7/12) if the focusing was proper. The middle portion of the curve (5/12 to 7/12 depth) approaches a straight line, hence any slight error in focusing creates but a slight error in electrophoretic velocities observed. Furthermore organisms are most easily seen at that depth at which they move fastest electrophoretically, i. e., at the depth 1/2. For these two reasons the writers have presented data as maximum values. It is true that such values are the sum of electrophoretic and of "return-flow" electroendosmotic velocities, and are only relative values. They seem, however, to be perfectly proper values to report in a comparative study, provided the "return-flow" electroendosmotic velocity is always the same at any depth under consideration, which should be the case provided the bacteria are always suspended in the same suspension medium (water in this case) in which case the electrical double layer between glass and suspension medium should be constant, and provided the irradiation produces no change in composition of the dispersion medium, which in turn might cause a change in electroendosmotic velocity (an increase in pH has been observed). Again, since the sequence of the actual electrophoretic velocities in the main resembled that of the sequence of the relative (maximum) velocities, it might be argued that whatever change in electroendosmotic velocity was produced was not of any great consequence.

Count x dilution x depth x fraction of sq. mm. x cu. mm. per cu. cm. = bacteria per cc., therefore $4 \times 20 \times 10 \times 400 \times 1,000 = 320,000,000$.

*This table compiled from data obtained with the technical assistance of Mrs. Sara W. Phillips.

In order to report actual electrophoretic velocities, readings were made at depth 1/6 in the Northrop Kunitz cell, a depth located on a very steep part of the Smoluchowski curve. It follows that any slight variation in focusing will bring about a rather large error in electrophoretic velocity. Since this value is multiplied by 3/4 whereas the value at 1/2 is multiplied only by 1/4, it follows that any slight error in focusing at 1/6 will make a relatively large error in the actual values reported. Besides, organisms are more difficult to see at depth 1/6 because they move so slowly especially when they have been rayed for a time sufficient to decrease their charge considerably. It is for this latter reason especially that the writers were prompted to abbreviate Table V.

B. STORAGE AND EXPOSURE DO NOT AFFECT ELECTROPHORETIC READINGS

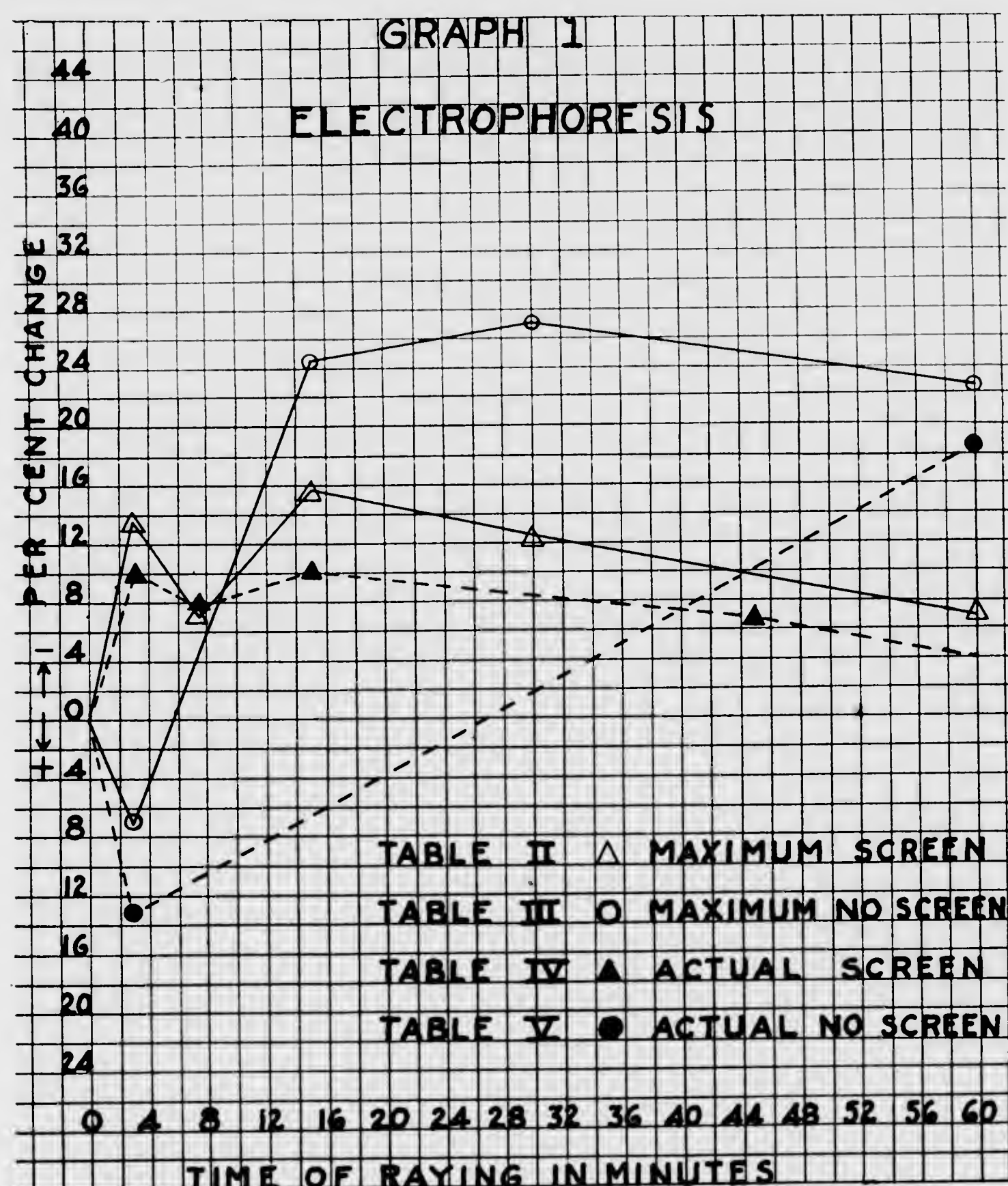
From Tables II to V inclusive it can be seen that storage of unrayed bacterial suspension was not the cause of changes in electrophoretic velocity reported. The average of maximum values of stored non-irradiated samples was but 1.5 per cent less than that of non-irradiated samples, and that of actual values but 2.5 per cent less, values considered within limit of error of 3 per cent previously reported by Tittsler and Lisse (1928). This means that the suspension can be prepared in large quantities, and if stored in the ice-box in rubber-stoppered Erlenmeyer flasks (the authors, in unpublished data, have found that cotton plugs will not always suffice) it can be used subsequently for studies on the effect of irradiation.

In similar unpublished studies one of us (Lisse) has found that if a sample (unrayed) was placed in the Northrop Kunitz cell and readings were made every five minutes over a period of four hours, the current being reversed every five minutes, the readings (average of 10 stop watch readings) varied only from 5.26 sec. to 5.48 sec. This means that any change observed as a result of irradiation of the suspension must have been due to the irradiation, directly or indirectly.

That the change in the electrophoretic velocity of irradiated samples was not due to the effect of any gases produced in the atmosphere during irradiation, or to the effect of any gases originally present in the atmosphere can be gleaned from Tables II to V inclusive. The average maximum value of "non-irradiated and exposed" samples was but 2.8 per cent less than that of non-irradiated samples, and that of actual values only 3.5 per cent greater. This means that exposure to atmosphere of the laboratory during the process of irradiation, for times similar to time of irradiation of the samples rayed, had practically no effect on electrophoretic velocity.

C. A CHANGE IN POTENTIAL DIFFERENCE ACCOMPANIES IRRADIATION.

That ultraviolet irradiation did change electrophoretic velocity can be seen in Tables II to V, and Graph I.



These data indicate that the effect might be stimulative initially, later becoming destructive. This is indicated by an initial slight increase in electrophoretic velocity followed by a much greater decrease as the irradiation is prolonged.

The data, however, show some discrepancies. When the Corex screen was inserted into the carbon arc lamp the initial stimulative effect was not observed, but only a destructive one. This suggests that rays coming from a carbon arc are of two types, stimulative and destructive, and that these play their part initially to varying degrees depending on quality and intensity of the irradiation.

That there might be an initial effect which is different from the later effect and therefore that there might be an initial stimulation accompanied by a different effect on electrophoretic velocity is suggested by results of Barnard and Morgan, Beaver and Muller, Benoit and Helbronner, Higgins and Sheard, Le Bon, Mond, O'Donnell, and Pech. Among these references can be found the idea that some light waves are bactericidal whereas others are therapeutic or physiologically active; that there is an interfering effect of some light waves on others, and that this effect should make itself felt when a soil or bacterial suspension is rayed for varying lengths of time. These findings suggest a repetition of this study, a repetition in which the bacterial suspensions are rayed with definite small bands of rays of equal energy content.

It is quite certain from the above tables that long irradiation has a charge-reducing effect as observed in a decrease of electrophoretic velocities. Witness decreases of 27 and 18.4 per cent in Tables III and V respectively.

Since reductions in electrophoretic velocities were greater when no Corex screen was used than when one was used this suggests that shorter wave lengths were more charge-reducing than longer ones. It is well known that when such screens solarize, the shorter wave lengths of ultraviolet are absorbed to a greater extent than by a non-solarized screen, and that even the latter does not permit all the ultraviolet rays produced by a carbon arc to pass through it. Hence one would expect to get a greater percentage of shorter rays, which are more bactericidal, when the screen is not used, and thus might expect a greater charge-reducing effect under such conditions, on the basis of the hypothesis that bactericidal and charge-reducing phenomena parallel each other.

D. CRITICAL DISCUSSION OF THE POTENTIAL AFFECTED BY IRRADIATION, AND MEASURED ELECTROPHORETICALLY.

A decrease in electrophoretic velocity, suggesting a decrease in potential difference, might be anticipated from numerous references in the literature.

It might be instructive to analyze these suggestions, for theoretically they do not suggest the same thing, even though they do suggest a decrease in potential difference. Let it be recalled that according to many modern colloid chemists, electrophoresis measures only the zeta potential, an electrokinetic potential, residing in a Helmholtz double layer. Besides this potential, bacteria, as well as all other cells, are known to possess a membrane potential, a thermodynamic potential, due to a Donnan equilibrium.

The studies of Millikan, Clark, and Bovie suggest that in decreasing the charge the photoelectric effect is active, causing electrons to be emitted from the substances affected, thus causing precipitation of

proteins, and the loss of charge by a negatively charged particle. Along with this goes a disturbance or destruction of the oriented and electrically organized interfaces. According to many modern colloid chemists, these effects must be effects on the Helmholtz double layer, causing a change in zeta potential. Surely Millikan, Clark, and Bovie have not told us the whole mechanism by which this change of zeta potential is brought about. They have given us a very general viewpoint based on a correct scientific principle, but they have told us little of the actual mechanism by which the change of zeta potential, which modern colloid chemists say is changed when changes in electrophoretic velocity are effected, is brought about.

It must be quite evident, however, if the photoelectric effect is bringing about a decrease in negative charge on the part of the surface-concentrated proteins which absorb the rays, and thus is precipitating them, that such precipitation must be followed by a change in permeability of membranes in which the proteins reside, which in turn must be followed by changes in Donnan equilibrium (tending to destroy it), lysis, and death (if the process has been of sufficient extent).

The work of Jensen and of Loeb suggests that the decrease in charge observed is due to a change in Donnan equilibrium, brought about by a change in membrane permeability, probably due to precipitation of protein. This change permits a difference in concentration of ions, to which the Donnan equilibrium is due, to be lost due to diffusion. These authors clearly state that what we measure electrophoretically is the result of such a Donnan or membrane potential. But this is a thermodynamic potential, and incapable of being measured electrophoretically according to many modern colloid chemists. It is quite likely, however, that any such changes due to diffusion must make themselves felt in changes in zeta potential.

It must be quite evident, if changes in potential which irradiation brings about are primarily thermodynamic potentials, that such changes must be accompanied by changes in permeability, lysis, and death (if the process has been of sufficient extent) [see Crile, Rowland and Telkes (1928)], and these in turn must be accompanied by changes in electrokinetic or zeta potential.

Let us refer again to the chapter by Lillie. If radiation is the stimulus which brings about a change in potential of the thermodynamic type, and if this is followed by a change in permeability, it seems logical to expect an ionic diffusion to result, one which should change the condition of the Donnan equilibrium. If this is so, then one would expect this change in ionic concentration to change the Helmholtz double layer and thus the electrokinetic potential, which change would make itself felt in a change in electrophoretic velocity. Thus it appears again that a change in thermodynamic potential of a cell will likely be accompanied by a change in its electrokinetic potential.

This critical discussion suggests the need of a very fundamental attack of the mechanism by which light brings about its changes, with a careful analysis of the relationship of the thermodynamic potential of a cell to its electrokinetic potential.

E. ELECTROPHORETIC CHANGES ARE PARALLELED TO A CERTAIN EXTENT BY CHANGES IN AGGLUTINABILITY, AND APPEAR MORE SENSITIVE THAN AGGLUTINATION STUDIES.

Since the work of Tittsler and Lisse and of others has called attention to the fact that the sequence of decreasing electrophoretic migration velocities followed the sequence of increasing agglutinability, it was only natural to look for the same relationship when the electrophoretic velocity was changed by irradiation with ultraviolet light.

Table VI shows that the effect of the Corex filter was to prevent that action from taking place which makes for greater agglutinability. In this case irradiation had little or no effect as measured by agglutination studies. Let it be recalled that in the electrophoretic studies the effect of the screen was to decrease the effect of the radiation to a large extent. Changes in electrophoretic velocity beyond the limit of error were observed however even when a screen was used. Since in general these changes were not apparent in the agglutination studies, it is suggested that in this work the electrophoretic velocities are more accurate measures of charge than studies of agglutinability. Agglutination is a fair method, however, by which to study changes in electrophoretic velocity, when these changes are sufficiently large, as will be shown immediately.

Again, the agglutination method is not apt to be as accurate as the electrophoretic method as a measure of charge because variations in standardization which will always appear will have an effect on agglutination values but not on electrophoretic values. Again, very slight differences can be observed electrophoretically but in agglutination studies such small differences are not detected especially in the usual procedure in which each successive dilution contains just half the serum-concentration of the previous one. Agglutinability depends on this concentration as well as on the charge.

From Table VII it becomes apparent that the previous conclusion of Tittsler and Lisse relative to the relation between electrophoretic velocity and agglutinability holds also when the samples were made to have a different charge by irradiation with ultraviolet rays, provided the irradiation was long enough. It might be suspected, if bacterial suspensions act as colloidal suspensions, that any difference in electrophoretic potential must make itself felt in a different agglutinability, the less the potential the greater the agglutinability. This conclusion was also verified by Mudd (1928). See Mattson (1929) in this connection.

F. EXPOSURE DOES NOT INTERFERE WITH AGGLUTINATION STUDIES.

It is evident from Table VIII, that the effect of ultraviolet rays on bacteria as studied by agglutination was really an effect of the radiation since agglutination data for samples that were exposed to the atmosphere of the laboratory in which the irradiations were carried out (but were not irradiated) were practically the same as those for unrayed samples.

G. LYSIS ACCOMPANIES IRRADIATION

That lysis has taken place during irradiation with ultraviolet light is apparent from Table IX in which 64 per cent of lysis was reported for 60 minutes of irradiation, the effect increasing with time.

Since lysis has taken place, the authors would expect a change in Donnan equilibrium (as a result of diffusion) to have taken place, and furthermore would expect this diffusion to have brought about a change in dispersion medium which in turn would have affected the zeta or electrokinetic potential, a change which would be measureable electrophoretically.

H. A DECREASE IN POTENTIAL ACCOMPANIES LETHAL ACTION OF ULTRAVIOLET IRRADIATION.

Two minutes of irradiation under conditions of the experiments was ample time to produce a sterile suspension, hence the conclusion that the bactericidal effect of ultraviolet irradiation is accompanied by reduction of electrophoretic velocity (charge) is justified. This relation between charge and viability might be compared with the unpublished data of Tittsler and Lisse which show that charge and activity of the nitrogen fixing organism parallel each other (high charge, high nitrogen fixing ability; low charge, low ability), and also with much of the work of Falk and his coworkers who showed, in a study of the pneumococcus, that charge and virulence are related properties. The work of Osterhout (1922) on conductivity studies in which he reports that when the organism is killed there is a loss of semipermeability also suggests that this should make itself felt in a change of electrophoretic velocity. Death, then, not only means an irreversible change in permeability but also a decrease in electrophoretic velocity (charge) which is paralleled by a greater agglutinability. [For the fact that aging and death are phenomena accompanied by decrease of thermodynamic potential approaching zero see Scurti and Cortese (1927) and Crile, Rowland and Telkes (1928)].

Summary

From this study of the effect of irradiation of suspensions of *Esch. coli* with the rays from a carbon arc, using B carbon electrodes, the following conclusions are drawn:

1. The Northrop Kunitz cell lends itself to electrophoretic studies of the effect of ultraviolet irradiation on washed aqueous bacterial suspensions.

2. Reasons are given for reporting relative or maximum values as well as actual values of electrophoretic velocities obtained with the Northrop Kunitz cell.

3. Storage and exposure to atmosphere of the laboratory in which the irradiation was carried out did not affect potential difference as measured by electrophoresis and by agglutination, and therefore were not the cause of the changes observed.

4. The insertion of a Corex A glass filter lessened the action of ultraviolet radiation considerably as measured electrokinetically and by agglutination.

5. Data obtained with the Northrop Kunitz cell, both relative and actual, indicate an initial stimulative action of ultraviolet radiation, one which makes itself felt in an increase of negative charge, and a lethal action which is accompanied by a decrease in charge as measured by electrophoresis.

6. Attention has been called to the confusion that has arisen because electrokinetic and thermodynamic potentials have not been differentiated carefully.

7. Electrophoretic studies are paralleled to a certain extent by changes in agglutinability and appear more sensitive than the usual agglutination studies for measuring the charge after irradiation.

8. Irradiation of *Esch. coli* produced lysis. This effect was greater the longer the irradiation.

9. Two minutes of irradiation under the conditions of the experiment was ample time to sterilize the bacterial suspensions.

PART TWO

Repetition of Part One, with Especial Emphasis on Short-time Irradiation

The Problem

The data reported in Tables III and V for 2, 3 or 4 minute irradiation indicate 7.2 and 13.2 per cent increase in electrophoretic velocity (charge). Since two minutes of irradiation was already sufficient time to sterilize the bacterial suspensions, it was thought advisable to make a detailed study of the change in electrophoretic velocity under irradiation as brief as 30 seconds, and to perform sufficient experiments, by the technique previously used (using no screen), to test the hypothesis that irradiation produces an initial stimulative action (one which makes itself felt in an increase of negative charge) and later a lethal action (accompanied by a decrease in charge), and that these changes can also be demonstrated by agglutination studies for changes in electrophoretic velocity are paralleled by changes in agglutinability. A record of the bactericidal effect of very short irradiation was also obtained. The data are presented in Tables X to XIII inclusive.

Experimental Results

TABLE X. AVERAGED MIGRATION VELOCITIES (IN MICRA PER SEC. PER VOLT PER CM.).
MAXIMUM VALUES (AT DEPTH 1/2) USING NO SCREEN. SEE GRAPH 2.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	21	23.13	
Irradiated 30 sec.	6	26.38	+14.1
Irradiated 1 min.	4	23.51	+ 1.7
Irradiated 1½ min.	2	23.00	— 0.6
Irradiated 2 min.	6	21.48	— 7.1
Irradiated 15 min.	8	15.07	—34.8
Irradiated 30 min.	9	14.72	—36.4
Irradiated 60 min.	1	11.68	—49.5

TABLE XI. AVERAGED MIGRATION VELOCITIES (IN MICRA PER SEC. PER VOLT PER CM.).
ACTUAL VALUES ($V=3/4$ v' at $1/6 + 1/4$ v' at $1/2$) USING NO SCREEN.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	8	10.53	
Irradiated 30 sec.	3	12.79	+21.5
Irradiated 1 min.	2	10.76	+ 2.2
Irradiated 1½ min.	2	11.82	+12.3
Irradiated 2 min.	2	10.42	— 1.0

TABLE XII. AGGLUTINATION vs. RAYING.

Antigen	No. of set-ups averaged	Serum Dilutions									
		40	80	160	320	640	1280	2560	5120	10240	20480
Non-irradiated	13	5	5	5	4	4	2	0	0	0	0
Irradiated 30 sec.	5	5	5	5	5	4	2	0	0	0	0
Irradiated 1 min.	5	5	5	5	5	5	4	2	0	0	0
Irradiated 1½ min.	5	5	5	5	5	5	4	2	0	0	0
Irradiated 2 min.	5	5	5	5	5	5	5	2	1	0	0
Irradiated 60 min.	4	5	5	5	5	5	5	5	5	5	½

Table XII gives the average of many agglutination set-ups which were designed to bring out the effect of short-time raying on agglutinability. No Corex filter was used, the raying was done at 30°C., the suspension was made to volume, 1/4 per cent formalin was added to the bacterial suspension, and readings were made after 48 hours incubation at 37°C.

That ultraviolet light did kill the organisms in a very short time is shown by Table XIII, which gives a record of sterilization with time.

TABLE XIII*. RECORD OF BACTERICIDAL EFFECT.

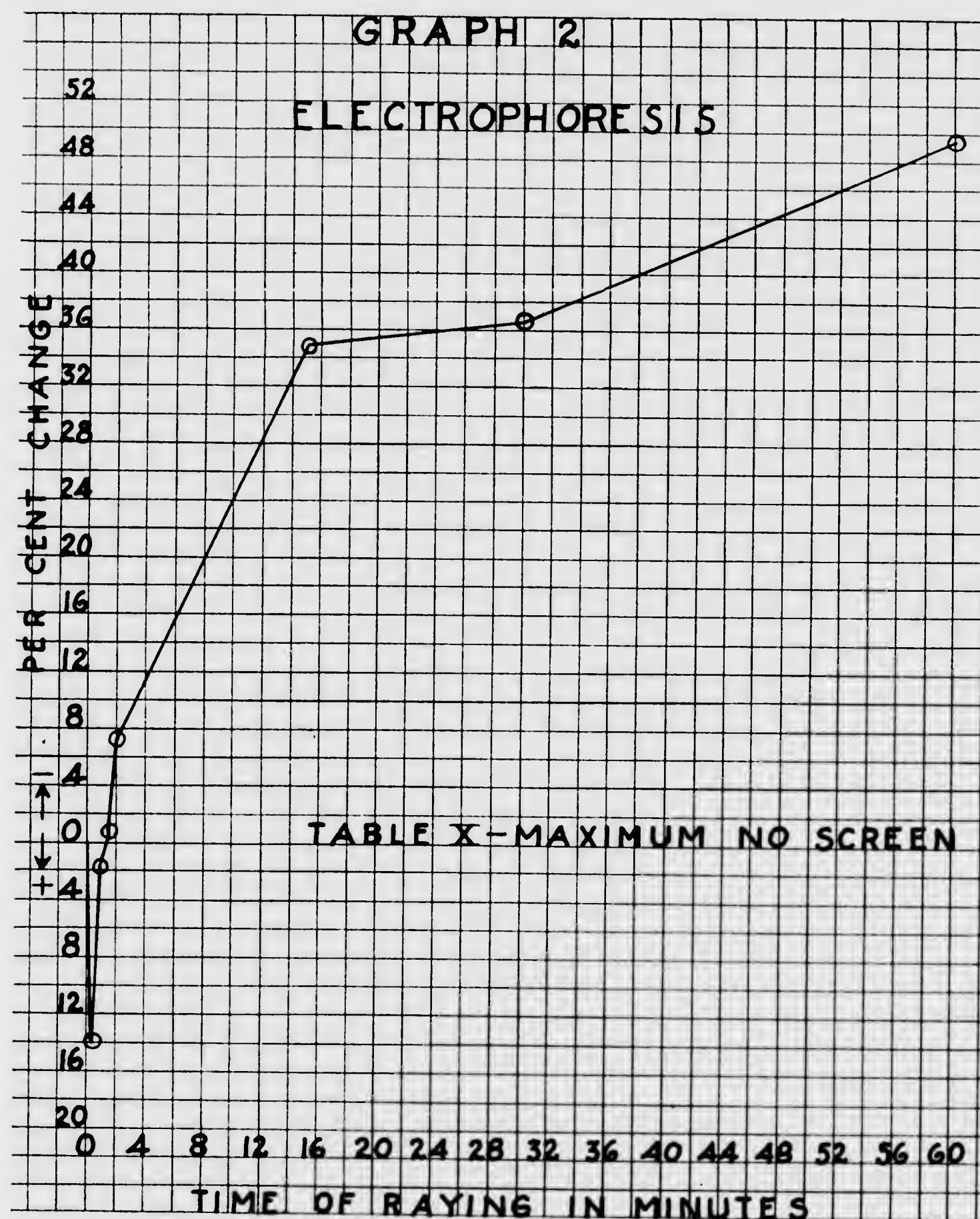
Treatment	Count per cc.
None	225,000,000
30 sec.	1,000,000
1 min.	50,000
1½ min.	few
2 min.	sterile

Discussion of Results and Conclusions

That ultraviolet irradiation of aqueous suspensions of *Esch. coli* did affect electrophoretic velocities is again shown in Tables X and XI and in Graph 2. These indicate that the effect is stimulative initially, later becoming destructive. This is observed by initial increases of 14.1 and 21.5 per cent and by the greater subsequent decrease of 49.5 per cent.

*This Table compiled from data obtained with the technical assistance of H. W. Williams.

It is of interest to observe that in PART ONE, Table III, a maximum migration velocity of 20.26 has been reported for non-irradiated samples and that in this work a value of 23.13 has been obtained, a rather fair agreement considering that values as low as 11.68 have been reported in Table X. A still better agreement is observed in



the actual values for the non-irradiated samples; witness 10.99 and 10.53 from Tables V and XI respectively. This indicates a tendency

for the strain of *Esch. coli* to maintain a rather constant electrokinetic potential on transplantation from slant to slant over a very long period of time as judged from the electrophoretic velocity of suspensions prepared from slants of a 24-hour incubation period.

An average of data presented in Tables III and X yields Table XIV.

TABLE XIV. MIGRATION VELOCITIES (IN MICRA PER SEC. PER VOLT PER CM.) MAXIMUM VALUES (AT DEPTH $\frac{1}{2}$) USING NO SCREEN. AN AVERAGE OF TABLES III AND X.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	33	22.69	-----
Irradiated 2, 3 or 4 min.	12	21.60	— 2.2
Irradiated 15 min.	11	15.15	—31.42
Irradiated 30 min.	12	14.74	—33.27
Irradiated 60 min.	5	14.91	—32.50

Table XIV again verifies the statement that after death by irradiation with ultraviolet rays there is a rather large decrease in zeta potential as measured by electrophoretic velocity.

If data from Tables V and XI are combined, Table XV results.

TABLE XV. AVERAGED MIGRATION VELOCITIES (IN MICRA PER SEC. PER VOLT PER CM.). ACTUAL VALUES. A COMBINATION OF TABLES V AND XI.

Treatment	Number of averaged averages	Migration velocity	Per cent change
Non-irradiated	17	10.77	-----
Irradiated 30 sec.	3	12.79	+18.8
Irradiated 1 min.	2	10.76	— 0.1
Irradiated 1½ min.	2	11.82	+ 9.7
Irradiated 2, 3 or 4 min.	5	11.63	+ 8.0

Table XV again verifies the statement that brief irradiation with ultraviolet rays stimulates, indicated electrophoretically by an increase of negative zeta potential normally carried by *Esch. coli*.

From Table XII it is apparent that the relation between electrophoretic velocity and agglutination seems not to exist except when non-irradiated antigen is compared with one irradiated 60 minutes. In the experiments of short-time irradiation, agglutination data point to a decrease in electrophoretic velocity which was not the case either for maximum or actual electrophoretic values obtained. Since in the determination of agglutinability (37° incubation) it becomes necessary to wait at least 24 hours before readings can be made whereas in the electrophoretic studies readings can be made within less than an hour after irradiation, a possible explanation of the discrepancy appeared in the idea that bacteria, irradiated but a short time and not receiving a lethal dose, have the power to return toward normal, i. e., to recover from the effect of irradiation. This idea prevailed in planning experiments cited in PART THREE. Again it appears that electrophoretic studies are more sensitive than agglutination studies (as usually made) for measuring the charge after irradiation with ultraviolet rays.

Two minutes, which was ample time to sterilize bacterial suspensions used in PART ONE, happens to be just the time necessary to kill all organisms as shown by Table XIII.

Summary

The data of PART TWO, serving as a repetition of PART ONE, point to the following conclusions:

1. Conclusions 5, 7, and 9 of PART ONE have been verified. Both relative and actual electrophoretic velocities of *Esch. coli* suspensions indicate an initial stimulative action of ultraviolet radiation (which makes itself felt in an increase of negative charge), and a lethal action (which is accompanied by a decrease in charge). Though electrophoretic studies are paralleled to a certain extent by changes in agglutinability, they appear more sensitive than agglutination studies (as usually made) for measuring the charge after irradiation.
2. Electrokinetic velocities of suspensions of 24-hour growths of *Esch. coli* show that the organism has a tendency to maintain a rather constant charge when transplanted to the same medium over a long period of time.
3. The lack of agreement in the findings by electrophoretic velocity and agglutination studies of suspensions of *Esch. coli* irradiated for sub-lethal periods with ultraviolet rays suggests that from such sub-lethal irradiation the organism has an ability to recover, a process in which its electrokinetic potential has a tendency to return toward normal.

PART THREE

Repetition of Previous Work but Using the Falk Cell, and Paying Especial Attention to the Process of Recovery and the Change of pH.

Review of Literature

A. THE FALK CELL

Mooney (1924), using a capillary tube open at both ends, reported that the mobility of oil drops increases with time during the first 5 or 10 seconds after the electric field is applied.

In the Northrop Kunitz cell endosmotic streaming along the walls of the cell is compensated by reverse streaming along the axis. To eliminate the resulting velocity gradient in the water, Mooney (1927) designed a new cell in which the water moves through the capillary with uniform velocity over its cross section, the compensating reverse flow taking place elsewhere.

Falk, Jensen and Mills (1928) improved the Mooney cell. They state that the observed mobility of bacteria in this cell is the difference between the velocity of the endosmotic streaming of the water and the velocity of electrophoresis of the bacterium. If the composition of the medium does not change, the endosmotic streaming of the water is sensibly constant. Hence, the observed mobility of the bacterium varies inversely as its true mobility, and is approximately independent of its position in the capillary tube. Furthermore polarization is practically eliminated by the interposition of distilled water between the electrodes and the open ends of the capillary tube.

An isolated colony can be picked from the growth-medium with a clean loop, emulsified in a small quantity of water and the suspension used for electrophoretic studies.

B. PROCESS OF RECOVERY

The total time required to kill bacteria in suspensions or on an agar plate is not changed by discontinuous exposure according to Norton in Jordan and Falk (1928) and Bovie (1915, 1916). Results of germicidal action of ultraviolet rays on *B. coli*, by Coblentz and Fulton (1924), showed "but little, if any difference in density of the growth of bacteria whether the method of exposure was continuous or intermittent." Hence it would appear that intermittent exposure does not have a latent effect during the intervals of rest, either in stimulating growth or in continuing the lethal action, and that the lethal effect is additive even with rest periods many times longer than the exposure periods. This agrees with the conclusion as to the additive effect of Schumann rays made by Bovie (1915, 1916).

C. CHANGES OF pH DUE TO IRRADIATION

Hill (1924) reported that an increase in hydrogen ion concentration accompanies the aggregation of particles in the cells of infusoria and of bacteria observed under the microscope as the lethal action of the rays progresses. Lillie, p. 201 in Cowdry (1924), states that a stimulus operates by oxidizing the surface, thus producing acid.

D. CONDUCTIVITY STUDIES

Determinations of electrical conductivity of cells and tissues, and of the effect upon it of various external agents have been made by a considerable number of investigators, but the application of the method in an extensive manner to the investigation of cell permeability is due to Osterhout (1922).

Osterhout's studies with many kinds of injurious agents showed a gradual fall of resistance, preceded in some cases by a preliminary rise, until a certain minimum was reached which was indicative of the complete death of the tissue. The whole course of the death-change could be represented by a curve whose regularity and freedom from breaks shows that death does not occur at any given instant but is a progressive change, which may even be reversed in its earlier stages by a return of the tissues to normal conditions. See also Green and Larson (1922).

Shearer (1919, 1922) concludes that bacteria, unlike other cells, have their maximum resistance, and therefore their maximum impermeability initially, i. e., prior to the treatment with solutions of various ions.

The Problem

Since (a) the data on agglutination presented in Table XII did not conform to the previously made hypothesis by Tittsler and Lisse (1928) and suggested a recovery (toward normal properties) following sublethal irradiation, since (b) the Falk capillary cell is simpler, less costly and more rapidly operated, the latter permitting electrophoretic readings very soon after irradiation of bacterial suspensions and probably before recovery has taken place, since (c) Hill and our preliminary work indicated that a change in pH would result from irradiation and would accompany the changes in charge and agglutination previously observed (as well as the lysis and death), and since (d) Winslow, Falk and Caulfield (1923) showed that slight changes in pH were accompanied by rather large changes in electrophoretic velocities, the following questions were raised, the answering of which constitutes the problem studied:

1. Will the Falk capillary cell lend itself to a study of the effect of irradiation on aqueous bacterial suspensions as studied electrophoretically?

2. If so, what differences in electrophoretic velocity will be observed

if readings are made immediately, early, and late, and what evidence will these readings give for the idea of a recovery period?

3. Will agglutination data indicate a recovery period if the immune serum is added at different times after irradiation of the aqueous suspension of *Esch. coli*?

4. What change in pH, if any, accompanies the process of irradiation of the bacterial suspension?

5. Can the results previously obtained be duplicated?

Technique

A. MEASUREMENT OF ELECTROPHORETIC VELOCITY

Samples of *Esch. coli* were prepared and irradiated in the same manner as previously. Electrophoresis, however, was performed in Falk capillary cells. After filling, the capillary was placed on the hook electrodes in a bath filled to a definite depth with distilled water, and centered as nearly as possible. Measurements were made at $49.5 \pm .5$ volts at a magnification of 475 diameters.

It was observed, in harmony with the findings of Mooney, that if the current was allowed to flow a few seconds before taking the first readings, a more uniform set of data would be obtained, and a time lag would be overcome. In this work, however, the time lag was such that electrophoretic velocities decreased.

The actual readings were made soon after filling. Five readings were made with the current flowing in one direction and five with it flowing in the other direction. Thus ten individual stop-watch readings were made with three to four different capillaries on the same suspension and these 30 or 40 readings were averaged. Similar averages were obtained with three different suspensions under any one treatment. Values reported in Tables XVI to XVIII are the averages of 90 to 120 individual stop-watch readings.

The results obtained are also reported in these tables as per cent change from the unrayed suspension.

Since readings could be made with the Falk capillary cell so much sooner after raying than with the Northrop Kunitz cell, it was observed that in the short-time rayings there was a change in the electrophoretic velocity when the suspension had stood for a while after raying, and that the initial readings were in all cases different from the subsequent ones. Therefore, "unrayed average of 30" means average of 30 readings on the untreated sample, obtained as per Table XVI; "initial readings" the average of the readings taken with the first capillary used immediately after raying; "early readings" the average of the average of the readings made with three capillaries used immediately after raying; and "late readings" the average of the average of the readings with three capillaries after the suspension had stood for at least three hours after raying.

B. AGGLUTINATION

Agglutination tests were performed as described under PART ONE except that immune serum was added at different times after the irradiation of the bacterial suspension, at times comparable to the times after irradiation at which the "initial," "early" and "late" electrophoretic readings were made. Values reported for each treatment (Tables XIX and XX) are averages of duplicate determinations on three different suspensions and, therefore, of six different agglutination tests for each treatment.

C. LYSIS

All studies of lysis were made by using a Petroff-Hausser bacteria counter. The values reported in Table XXI are in per cent decrease from the original suspension. Each value for any treatment was obtained from an average of duplicate determinations on three different suspensions, and is therefore an average of six determinations on each treatment.

D. pH STUDIES

All pH determinations were obtained by the LaMotte method.

Experimental Results

A typical set of electrophoretic readings is presented in Table XVI, to show how closely results can be duplicated when the Falk capillary cells are used.

TABLE XVI. INDIVIDUAL STOP-WATCH READINGS ON A SINGLE SUSPENSION USING DIFFERENT CAPILLARIES. VALUES ARE IN SECONDS TO TRAVEL 220 MICRONS, VOLTAGE $49.5 \pm .5$ VOLTS.

Capillary, No. 1	Capillary, No. 2	Capillary, No. 3
11.56	11.81	12.06
11.26	11.06	11.60
10.60	10.23	11.67
11.05	9.84	10.81
11.44	10.82	10.35
	Current Reversed	Current Reversed
11.17	11.46	10.70
10.70	10.80	10.59
10.50	11.06	10.50
10.86	11.30	10.51
10.52	10.29	11.20
Average 10.97	Average 10.88	Average 11.00
	10.96 = average of 30 readings	

Table XVII shows a complete set of typical data for one treatment. Values in this Table are obtained in the same way as the average value in Table XVI.

TABLE XVII. AVERAGE OF OBSERVED READINGS AND PER CENT CHANGE, RAYING DIFFERENT SUSPENSIONS 30 SECONDS

Unrayed Av. of 30	Initial readings Av. of 10	Early readings Av. of 30	Late readings Av. of 30	Per cent change, initial	Per cent change, early	Per cent change, late
9.78	8.50	9.56	9.02	-14.83	-4.21	-9.62
10.07	9.65	10.09	10.03	-4.17	+0.20	-0.40
9.29	8.65	8.97	8.90	-6.89	-3.44	-4.20
Av. 9.78	8.93	9.54	9.32	-8.63	-2.48	-4.74

The final averages of all treatments are given in Table XVIII. These values have been obtained in the same manner as the average values given in Table XVII.

TABLE XVIII. FINAL AVERAGES OF OBSERVED READINGS AND PER CENT CHANGE UPON RAYING. SEE GRAPH 3.

Unrayed: Av. of 30	Treatment	Initial readings Av. of 30	Early readings Av. of 30	Late readings Av. of 30	Per cent change, initial	Per cent change, early	Per cent change, late
9.78	Rayed 30 sec.	8.93	9.54	9.32	-8.63	-2.48	-4.74
9.69	Rayed 1 min.	8.81	9.88	8.66	-9.08	+1.96	-10.63
9.99	Rayed 2 min.	8.72	9.07	9.10	-12.71	-9.21	-8.91
9.93	Rayed 5 min.	9.26	9.10*	9.10†	-7.35	-8.36	-8.36
10.38	Rayed 15 min.	8.54	8.58	8.58‡	-17.73	-17.34	-17.34
10.32	Rayed 30 min.	7.81	7.78	7.78‡	-24.32	-24.61	-24.61

All agglutination tests were run in duplicate on three different suspensions for each treatment. Averages for all treatments are reported in Table XIX. Each set of agglutination data reported is the average of three different sets of duplicates.

* Average of only 60 readings.

† Only sufficient data were taken to show that there was no change on standing.

‡ These values can be expressed as $10.0 \pm .4$.

TABLE XIX. AVERAGE OF AGGLUTINATION TESTS.

Treatment		Serum Dilutions						
Antigen	Immune serum added after storing	160	320	640	1280	2560	5120	10,240
Unrayed	0 min.	5	4	2	0	0	0	0
30 sec.	0 min.	5	5	5	1	0	0	0
30 sec.	40 min.	5	5	4	0	0	0	0
30 sec.	180 min.	5	5	4	1	0	0	0
1 min.	0 min.	5	5	4	1	0	0	0
1 min.	40 min.	5	5	4	1	0	0	0
1 min.	180 min.	5	5	4	2	1	0	0
2 min.	0 min.	5	5	5	3	1	0	0
2 min.	40 min.	5	5	5	4	3	0	0
2 min.	120 min.	5	5	5	4	2	1	0
5 min.	0 min.	5	5	5	5	4	1	0
5 min.	180 min.	5	5	5	5	4	1	0
15 min.	0 min.	5	5	5	5	5	2	0
30 min.	0 min.	5	5	5	5	5	3	0

The results of studies on lysis by the direct count method are given in Table XX. Data give per cent decrease in count, the unrayed suspension being taken as a standard, each value being the average of 180 readings (counts).

TABLE XX. THE EFFECT OF RAYING ON LYSIS.

Treatment	Per cent decrease
Rayed 30 sec.	13.9
Rayed 1 min.	24.0
Rayed 5 min.	37.7
Rayed 15 min.	56.9
Rayed 30 min.	63.7

The results of the pH determinations (La Motte method) are shown in Table XXI.

TABLE XXI. pH DETERMINATIONS

pH of unrayed suspensions	Treatment	No. of determinations	pH of treated samples
6.1 ± .1	Rayed 30 sec.	4	6.8 ± .2
6.1 ± .1	Rayed 1 min.	2	6.9 ± .1
6.1 ± .1	Rayed 5 min.	3	7.0
6.3 ± .2	Rayed 15 min.	2	7.2 ± .2
6.1 ± .1	Rayed 30 min.	2	7.2 ± .1

Discussion of Results

A. ELECTROPHORETIC READINGS WITH FALK CELL. ARE THEY TRUSTWORTHY?

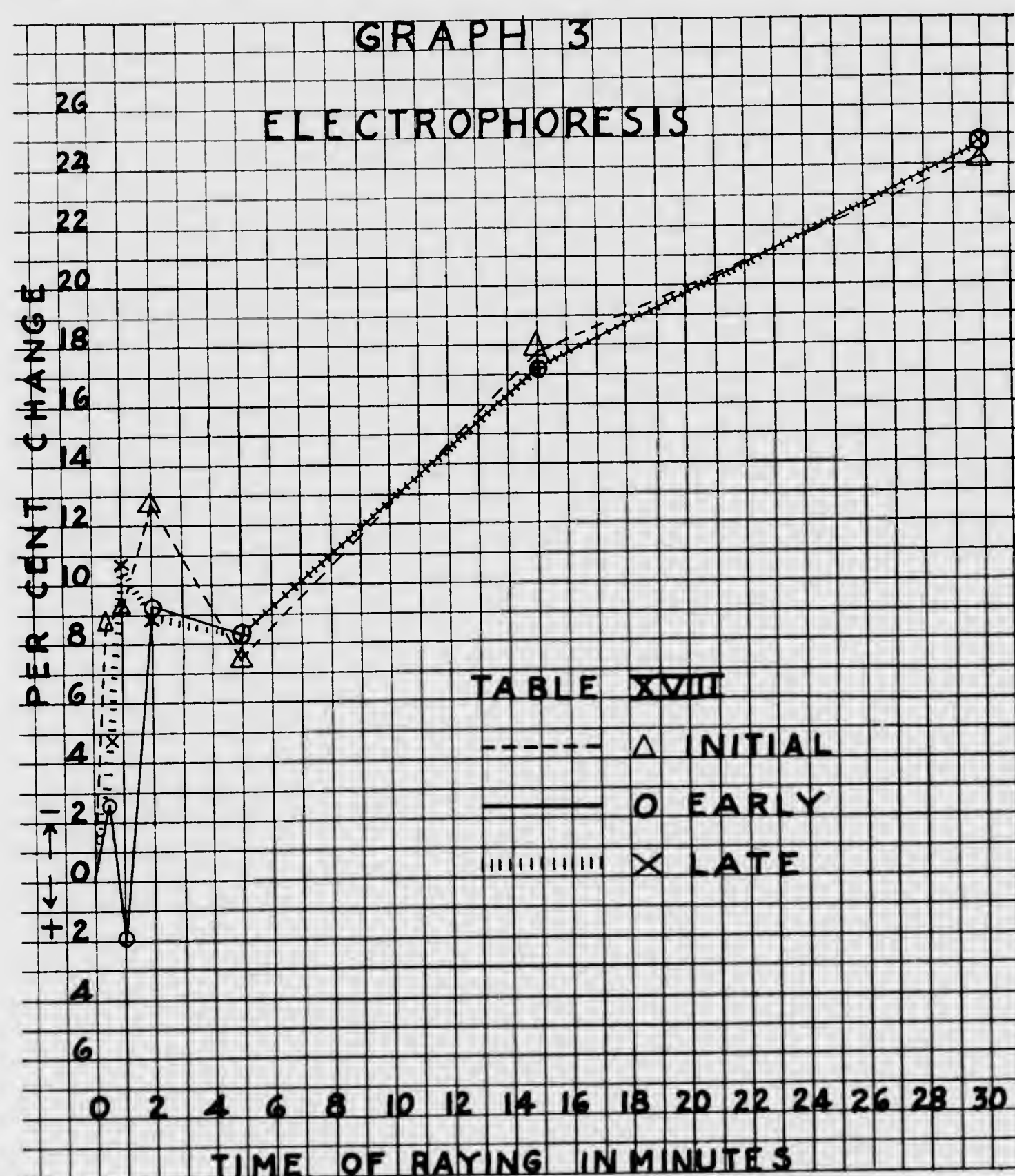
Table XVI indicates that results can be duplicated very well when the Falk capillary cell is used. This is not true when capillaries are chosen at random. For this work capillaries were chosen which gave practically identical results when used with aqueous suspensions of *Esch. coli* at a definite pH.

In some data for brief irradiation (Table XVII) the agreement was not quite so good, perhaps due in part to the fact that readings were not always made at exactly the same point in the recovery period, a period suggested by the data obtained.

It was because readings with the Falk cell can be made so much sooner after irradiation than with the Northrop Kunitz cell, (one may focus anywhere except near the surface and filling of cell is more readily accomplished), and because with it a recovery period (suggested by Table XII) might more easily be demonstrated, that it was thought to be a more desirable type of cell for use in studies of the effect of irradiation on the electrophoretic velocity. On the other hand, since pH changes (Table XXI) accompanied irradiation, and since such changes might affect both electroendosmotic and electrophoretic velocities, it might appear that the values obtained in Table XVIII are useless, unless it can be shown that electroendosmotic velocity remains constant and is not affected by changes in pH.

That the results obtained with the Falk cell are in the same direction as in the previous work for the longer times of irradiation is shown by the figures —24.2, —34.8, and —17.34 for 15 minute irradiation and —27.0, —36.4, and —24.32 for 30 minute irradiation (see Tables III, X and XVIII respectively), and also by the fact that from Table XVIII we again find that as irradiation continues after death the negative charge carried by *Esch. coli* is more and more decreased.

It is with brief irradiation that the agreement in values obtained with the Falk and Northrop Kunitz cell is rather poor. The increase in charge indicating a stimulation at the start of irradiation is almost entirely missing. The conclusion is drawn, therefore, that the Falk



cell will not lend itself with certainty to a study of the effect of ultraviolet irradiation unless it can be shown that changes of ionic environment produced by irradiation affect the cataphoretic velocity only, or unless it can be shown that determinations of actual velocity made with the Northrop Kunitz cell are in the same order as values obtained with the Falk cell.

B. INITIAL, EARLY, AND LATE ELECTROPHORETIC READINGS SUGGEST A RECOVERY PERIOD

A study of data obtained in PARTS ONE and TWO led us to suspect that the amount of time elapsed between the process of irradiation and the making of electrophoretic readings was a factor which must be taken into consideration in analyzing variations obtained. It was thought, if Osterhout's observations on conductivity were correct, that a similar effect was to be expected in electrophoretic velocities. For instance, if the cell was merely injured by very short irradiation it might have recovery power after the stimulus is taken away, and thus a tendency for the electrophoretic velocity to return to that of the unirradiated sample, but if the cell was killed, such power of recovery would not be present. If such were the case one would expect greater variations in the electrophoretic velocities in immediate ("initial"), and later readings (at least in "early" and perhaps in "late") when suspensions were used which were irradiated less than the time necessary to kill than when those which were rayed for a longer time were used. Furthermore one would expect a return to normal in studies involving short-time irradiation and no such return (constant readings) in studies in which the suspensions were irradiated for a longer time.

That this hypothesis has more than a germ of truth in it is suggested by the data given in Tables XVII and XVIII. When the suspension was irradiated for only 30 seconds, witness the change in velocity from 8.93 for initial to 9.54 and 9.32 for later readings, 9.78 being the value for the non-irradiated sample. Note also that values for velocities of samples rayed 15 or 30 minutes were constant, showing no return to normal. In Table XVIII, the fact that the values 9.54 and 9.88 for "early" readings are followed by 9.32 and 8.66 respectively for "late" readings suggests that even with 30 seconds and one minute irradiation the recovery just mentioned might not be a permanent one. This will be discussed in detail in a subsequent paper.

These values not only indicate the need of making readings at a definite time after irradiation especially when the irradiation time is short, provided one desires to obtain comparative readings in a comparative study of the effect of irradiation on bacterial electrophoretic velocities, but they also suggest that short-time irradiation merely injures cells (Osterhout) i.e., brings about a temporary abnormal condition, and a reversible change in permeability permitting recovery, while long-time irradiation, causing death, brings about an irreversible change in permeability from which the cell cannot recover.

C. AGGLUTINATION CAN NOT ACCURATELY MEASURE EFFECT OF SUB-LETHAL IRRADIATION AND INDICATES RECOVERY PERIOD

Like Table XII, Table XIX suggests that the initial stimulation which was observed in an increase in electrophoretic velocity (Tables III, V, X, XI) is an erroneous observation (see Summary, 5, PART

ONE). It suggests that the decrease in charge is experienced even with brief irradiation, or that even here it follows the stimulatory increase.

That there is a recovery period, and that agglutination does not accurately measure the effect of sub-lethal irradiation is suggested by the fact that electrophoretic velocity differences were observed in "initial," "early" and "late" readings of Table XVIII, whereas agglutination data obtained (Table XIX) when the immune serum was added at times comparable to the times at which the "initial," "early," and "late" readings were made, showed no significant differences.

D. LYSIS ACCOMPANIES IRRADIATION

Table XX is comparable to Table IX.

E. AN INCREASE OF pH ACCOMPANIES IRRADIATION

Changes in pH were frequently observed accompanying irradiation of bacterial suspensions during the work of PARTS ONE AND TWO, but a comprehensive study of this change was made in connection with this work only. The data are reported in Table XXI which shows that, even with 30 seconds irradiation, there was an increase of pH of approximately 0.7, increasing to 1.1 for irradiations of 30 minutes. Since the pH of the water used was 6.0 to 6.3, and that of suspensions prepared also 6.0 to 6.3, irradiation was accompanied by an increase in pH, i. e., by a change to alkalinity.

These data are contrary to the expectations of the authors, Hill having shown that an increase in hydrogen ion concentration accompanies the aggregation of particles in the cells of infusoria and bacteria observed under the microscope as the lethal action of light progresses, and Lillie having suggested that a stimulus operates by oxidizing the surface, thus producing acid.

Since there has been an increase in pH it occurred to the authors that this increase might have been brought about by diffusion outward, during change of permeability or during lysis, of cell contents of pH higher than that of the dispersion medium, and that this change of ionic concentration was responsible for the change in electrophoretic velocity. That this is not the explanation for the increase in pH observed will be shown in a subsequent paper.

Summary

1. The Falk cell lends itself to obtaining practically identical results with capillaries of the same length, etc.
2. Electrophoretic velocities indicate a recovery period following sub-lethal irradiation.

3. With lethal periods of irradiation, a decrease in charge accompanies death of *Esch. coli*.

4. Agglutination can not accurately measure the effect of sub-lethal irradiation, and indicates a recovery period.

5. Lysis and increase of pH accompany irradiation.

6. A time-lag, pointed out by Mooney, was observed by electrophoretic measurements.

General Summary

The hypothesis is made that irradiation of *Esch. coli* suspensions with rays from a "B" carbon arc should effect (a) changes in the electrophoretic velocity and charge of the organism, (b) changes in the agglutinability, (c) lysis, (d) changes in viability, and (e) changes in pH, and that the irradiation should produce an initial stimulative action, one which makes itself felt in temporary increase of negative charges, and a lethal action which is accompanied by a permanent decrease in charge measured electrophoretically, and that these changes should be paralleled, at least to a certain extent, by changes in agglutinability.

The extent to which the data obtained in three separate researches agree with the above hypothesis, and various other findings are given in three separate summaries. (See pages 24, 30, and 40).

Acknowledgment

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THE INCORPORATION OF SCIENTIFIC SOCIETIES

At the Cleveland 1930-31 meeting of the American Society of Plant Physiologists the question as to the advisability of incorporating the society was referred to a committee.

Of the twenty-one scientific societies investigated, fifteen are incorporated. Seven of these societies are incorporated in the District of Columbia, one in Illinois, one in Wisconsin, one in Massachusetts, one in New York and one in Pennsylvania. As the reasons for and ways of incorporating learned organizations may be of interest to other scientific societies the findings of the above committee are here summarized.

The advantages of incorporation are important, inasmuch as it establishes a learned non-profit organization as a legal entity, thus bestowing:

(1) Freedom from financial responsibility in any lawsuit against the members on account of any action of the society.

(2) The ability to hold property and to receive gifts and bequests.

A few scientific societies reported to our committee that incorporation had resulted in the accumulation of funds for research, etc., by giving greater security to endowment funds and thus making it easier for donors to give relatively large gifts and bequests.

The possible disadvantages are related to the freedom of action of the organization. An unincorporated society may do as it chooses, whereas upon incorporation the organization must comply with the laws of

the jurisdiction where it is incorporated. Practically, however, the disadvantages resulting from this restraint or freedom of action is regarded by legal authorities as being slight. The statutes of some jurisdictions impose fewer restraints than others and the requirements for headquarters, annual meetings, reports, etc., differ in different jurisdictions.

The procedure of incorporating under the laws of the District of Columbia, and also in certain other jurisdictions, is relatively simple. The statutes of the District of Columbia relating to corporations and of certain other jurisdictions relating to learned *non-profit* organizations neither require a resident director nor the maintenance of an office in the jurisdiction. Meetings may be held anywhere and at any time without restriction.

The principal requirements under the laws of the District of Columbia may be taken as an example of the general procedure: (1) Any three or more persons of full age, citizens of the United States, the majority of whom are citizens of the District of Columbia may incorporate; (2) a certificate in writing must be filed in the office of the Recorder of Deeds stating (a) the name of the society, (b) the term for which it is incorporated, which may be perpetual, (c) the business and objects of the society, (d) the number of its trustees, directors or managers for the first year of the incorporated society's existence.

Upon the execution of the articles of incorporation of the society and the deposit of same with the recorder of deeds, the incorporators then form a temporary organization in Washington, D. C., at which meeting one of their number is elected temporary chairman and another temporary secretary. The constitution and by-laws of the organization as then standing are adopted, and all the then existing members of the society are declared elected members of the corporation with their present rights and privi-

leges. The meeting then adjourns to assemble at the next regular place of meeting of the now incorporated society.

Many of the scientific societies investigated dispensed with legal assistance. Under such conditions the only expense involved is the registration fee of two or three dollars. A few societies, however, obtained advice of counsel in making such changes in their by-laws to conform with the statute under which incorporation is sought. The expense in such cases is about one hundred dollars.

A comprehensive discussion of the business relations of non-profit corporations is given by Harriman in *Corporate Practice*, Review 1, 7-11, 1929.

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THE ELECTROPHORETIC POTENTIAL OF RHIZOBIUM MELILOTI^{1,2}

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It is generally accepted that strains of *Rhizobium meliloti* vary greatly in their ability to "fix" nitrogen. The species was divided into two serological groups by Stevens (1923). These groups, designated as A and B, also differ in other respects. Group A grows faster, produces a more abundant growth, is more sensitive to acid, and produces a greater change in titratable acidity and hydrogen-ion concentration of the medium than group B. Stevens (1925) reported that "in general the group A strains fixed almost twice as much nitrogen as did the group B strains." In view of these well defined and constant differences Stevens (1925) appears to be justified in his conclusion that "these variations are manifestations of inherent differences in the nature of the organisms." It seemed logical to us to assume that the "inherent differences" are of such a physico-chemical nature that they should be measurable by suitable methods. This suggested the possibility that differences in electrophoretic potential might exist in relation to the differences in nitrogen fixing ability. A direct relationship between the magnitude of electrophoretic potential and the degree of virulence of pneumococci was reported by Falk, Gussin and Jacobson (1925). During the progress of the present study,

¹ The experimental data obtained by the Northrop-Kunitz method are from a thesis submitted by R. L. Ferguson in partial fulfillment of the requirements for a degree of Master of Science in the Graduate School of The Pennsylvania State College, 1928.

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Jensen and Falk (1928) reported an inverse relationship between the electrophoretic potential and the toxigenicity of diphtheria bacilli.

The purpose of this investigation was to determine, first, if strains of *Rhizobium meliloti* vary in electrophoretic migration velocity and, second, in case variations were found, to note any relationship to nitrogen fixing ability. A preliminary report by Tittsler and Lisse (1928b) indicated a direct parallelism between electrophoretic migration velocity and nitrogen-fixing ability.

Zucker (1929) in a preliminary report of a similar study stated "that a certain relationship exists between acid production and electrophoretic velocity. In general the acid formers show a higher velocity than the non-acid producers. Usually the higher the velocity the poorer the cultures are with respect to their effect on the growth of the higher plant." Later, Zucker, Baldwin and Fred (1930) stated "there are only slight differences in the electric charge carried by various strains of the legume nodule bacteria, and these variations are not correlated with the ability of particular strains to fix nitrogen." Baldwin (1930) stated that "with the alfalfa group the good strains generally have a higher velocity than the poor strains and that just the opposite occurred with the soy bean group. With the other strains studied, it was impossible to make any correlation between the two points. In all cases the differences between strains were very slight."

Zucker (1930) came to the conclusion that "in the alfalfa group, the 'good' strains generally have higher velocities than the poor strains." He showed that the rate of migration velocity was altered by the medium upon which the organisms were grown, the degree of dilution of the cell suspension, the gum or slime and acid by-products, and the storage of the organisms in distilled water.

TECHNIQUE

Cultures. A total of 20 cultures was used in this study. With the exception of strains 1, 2 and 16 they were obtained from other investigators. Strains 5, 6, 7 and 8 were from the

group studied by Stevens (1925) who found considerably more nitrogen fixed in plants inoculated with strains 5 and 8 than in those inoculated with strains 6 and 7. Detailed information as to source, original designation and previous descriptions of all cultures is given in table 1.

Medium. The composition of the medium used was as follows:

Potassium phosphate, K_2HPO_4	1.0 grams
Calcium carbonate, $CaCO_3$	2.5 grams
Mannitol, $C_6H_8(OH)_6$	10.0 grams
Agar, Difco.....	20.0 grams
Distilled water.....	1,000 cc.

It was tubed, sterilized at 15 pounds pressure for twenty minutes and slanted.

Preparation of suspension. The agar slants were inoculated from fresh cultures grown on the same medium and were incubated for one week at room temperature. The growth was removed with a small volume of distilled water by a gentle rotary movement of the culture tube. This suspension was filtered through cotton, sedimented in a centrifuge at about 3,000 r.p.m. and the cells resuspended in distilled water. The washing of the cells was repeated twice more to remove traces of medium and metabolic products which may have been carried over from the agar. All suspensions were diluted to an arbitrary standard of turbidity and used for electrophoretic measurements as soon as possible on the same day they were prepared.

Measurements of electrophoretic velocities. Migration velocities in the Northrop-Kunitz (1924) cell were measured in essentially the same manner described by Tittsler and Lisse (1928a). Ten measurements of velocity (5 with one and 5 with reversed orientation of the electrical field) were made at 7/16, 8/16 and 9/16 of the depth of the cell. The cell was then refilled and another set of measurements was made. The two sets were averaged provided their differences was not greater than 4 per cent. In case of greater differences, further measurements were made. Thus, each value given represents the average velocity of at least 60 individual bacterial cells.

The capillary method described by Falk, Jensen and Mills

TABLE 1
History of cultures

STRAIN	RECEIVED FROM	ORIGINAL STRAIN	SOURCE	DATE ISOLATED	CHARACTERISTICS OR HISTORY FURNISHED WITH CULTURES
1	Pennsylvania State College	Stock	Alfalfa	1927	Considered efficient
2	Pennsylvania State College	97	Alfalfa		No plant tests
3	Michigan State College	8	Alfalfa	1925	"Gave positive inoculation"
4	Iowa State College	100	Alfalfa	1912	High nitrogen-fixing ability
5	University of Wisconsin	101	Alfalfa	1915	Low nitrogen-fixing ability
6	University of Wisconsin	102	Alfalfa	1916	Low nitrogen-fixing ability
7	University of Wisconsin	107	Alfalfa		High nitrogen-fixing ability
8	United States Department of Agriculture	225	Sweet clover	1914	Low nitrogen-fixing ability
9	United States Department of Agriculture	436	Sweet clover	1922	High nitrogen-fixing ability
10	United States Department of Agriculture	450	Alfalfa	1925	High nitrogen-fixing ability
11	University of Idaho		Alfalfa		"From root covered with nodules"
12	University of Idaho		Alfalfa		"A very efficient culture"
13	University of Illinois	Stock			
14	University of Illinois	A. R. M.			
15	Pennsylvania State College	3DOa10	Alfalfa	1928	Average nodule production
16	University of Maryland	3DOa9	Alfalfa	1925	"Gave good inoculation"
17	University of Maryland	3DOa2	Alfalfa	1924	"Gave very good inoculation"
18	University of Maryland	3DOh1	Sweet clover	1924	"Gave positive inoculation"
19	University of Maryland			1924	"No plant tests"
20	University of Maryland				

(1928) was used in the second portion of the study to determine whether the earlier results could be duplicated with another type of apparatus. Since the migration velocity in this cell is affected by the length and bore of the capillary, 3 capillaries which gave almost identical velocities in preliminary tests were selected for use in this study. Measurements were made upon 10 bacterial

TABLE 2
Migration velocities and history of all cultures
Type of electrophoresis apparatus

NORTHROP-KUNITZ			FALK CAPILLARY		
Strain	μ /sec. (60 volts)	Nitrogen-fixing ability or history	Strain	μ /sec. (45 volts)	Nitrogen-fixing ability or history
8	70.28	High	5	15.35	High
10	62.24	High	8	15.96	High
11	60.00	High	17	17.05	"Good inoculation"
7	53.23	Low	13	17.08	"Very efficient culture"
5	52.40	High	19	17.10	"Positive inoculation"
1	51.17	Unknown	4	18.25	Unknown
6	50.33	Low	20	18.36	Unknown
3	48.71	Unknown	18	18.82	"Very good inoculation"
4	45.66	Unknown	16	19.24	Average inoculation
2	43.18	Unknown	10	19.38	High
9	35.00	Low	7	20.20	Low
			14	20.43	Unknown
			9	20.58	Low
			15	20.73	Unknown
			12	23.91	"Many nodules"
			6	25.26	Low

cells (5 with each polarity of the electrical field) in each of the 3 capillaries and the velocities of the 30 cells were averaged.

The results of our investigation are expressed in terms of observed electrophoretic velocity (in micra per second) at 60 volts with the Northrop-Kunitz cell and at 45 volts with the Falk capillary cell.

EXPERIMENTAL RESULTS

The results of all electrophoretic measurements are summarized in table 2. The cultures are listed in their order of decreasing electrophoretic velocity as determined by the two methods.

Attention is called to the fact that the observed velocities reported for the Falk capillary method vary inversely with their true electrophoretic velocities. With the Northrop-Kunitz method the fastest velocity ($70.28\mu/\text{sec.}$) was 100 per cent greater than the slowest (35.0), and with the Falk capillary method the fastest (25.26) was 64.5 per cent greater than the slowest (15.35).

Table 3 summarizes the results obtained with the cultures of known comparative nitrogen-fixing abilities. With the exception of strain 5 in the Northrop-Kunitz method, the high nitro-

TABLE 3
Migration velocities or strains of known nitrogen-fixing abilities
Type of electrophoresis apparatus

NORTHROP-KUNITZ			FALK CAPILLARY		
Strain	$\mu/\text{sec.}$ (60 volts)	Nitrogen- fixing ability	Strain	$\mu/\text{sec.}$ (45 volts)	Nitrogen- fixing ability
8	70.28	High	5	15.35	High
10	62.24	High	8	15.96	High
7	53.23	Low	10	19.38	High
5	52.40	High	7	20.20	Low
6	50.33	Low	9	20.58	Low
9	35.00	Low	6	25.26	Low

gen-fixing strains had a greater negative electrophoretic potential than those of lower nitrogen-fixing ability.

The depressing effect of calcium chloride upon the migration velocity was shown in a single experiment. A suspension of strain 10, prepared as described above, was divided into two lots; to one was added sufficient calcium chloride to make a 1/110 M concentration while the other remained as a distilled water control. The migration velocity in the calcium chloride menstruum was reduced to approximately one-fourth that in distilled water.

DISCUSSION

In view of the fact that various strains of *Rhizobium meliloti* differ greatly in their rate, type and amount of growth, acid-producing ability, sensitivity to hydrogen ions, serological reac-

tions and ability to fix nitrogen in the host plant it is not surprising that they should also differ in their electrophoretic behavior as shown in table 2. The differences of 10 to 100 per cent in the migration velocities are interpreted as expressions of actual differences in the physico-chemical nature of the various strains. The accuracy of the methods has been tested many times in this and other investigations, using suspensions of *Rhizobium meliloti*, *Salmonella pullorum*, and *Escherichia coli*, and we agree with Falk, *et al.* (1925), (1928) and Chapman (1929) that, with uniform technique, the experimental error is not greater than 4 per cent.

A possible relationship between electrophoretic potential and nitrogen-fixing ability is suggested by a comparison of the migration velocities of the known high and low nitrogen-fixing strains (table 3). Both the Northrop-Kunitz and the Falk capillary methods showed that the high nitrogen-fixing strains (with the exception of strain 5, Northrop-Kunitz method only) had a greater negative electrophoretic potential than those of lower ability. Furthermore, with the Northrop-Kunitz method the average velocity ($61.64\mu/\text{sec.}$) of the good strains was 33.4 per cent greater than the average (46.19) of the poorer strains, and with the capillary method (velocity inverse to potential) the average ($16.9\mu/\text{sec.}$) of the good strains was 30.2 per cent less than the average (22.0) of the poorer strains. The general similarity of the sequences of decreasing electrophoretic potentials obtained by the two methods is rather significant in view of the fact that the capillary method was used by different persons eighteen months after the Northrop-Kunitz method.

Zucker (1930) in analysing his results emphasizes the fact that the variations among different strains were small, never over two micra per second. However, when his differences are considered in terms of percentages rather than as micra per second they appear more significant. Although his fastest velocity ($5.14\mu/\text{sec.}$) was only $1.66\mu/\text{sec.}$ more than the slowest (3.48) it was 47.5 per cent greater. Furthermore, the average velocity ($4.63\mu/\text{sec.}$) of his four known "good" strains was 20 per cent greater than the average (3.86) of the five "poor" ones, although the actual difference was only $0.77\mu/\text{sec.}$ With one exception,

which might be classified as an intermediate, the "poor" strains had a lower potential than the "good" ones. The validity of our assumption that these differences are significant when considered in terms of percentage is nullified in case an experimental error of equal magnitude is admitted. However, our experience with the Northrop-Kunitz and Falk capillary methods indicates that differences of 10 per cent or more are greater than the experimental error. Therefore, we consider the differences in the "true" velocities observed by Zucker at the "stationary levels" in the Falk (1928) slide cell as signifying actual differences in the electrophoretic potential of various strains.

The observations of Zucker and ourselves that, in general, the strains of higher efficiency had a greater negative electrophoretic potential than those of lower ability were not sufficiently extensive to warrant a final conclusion. Nevertheless, they do indicate a possible relationship which is certainly worthy of further investigation involving a greater number of strains, plant tests and nitrogen determinations. Such a study should also involve a comparison of electrophoretic potential and infectiveness (nodule-forming ability) since Allen and Baldwin (1931) assume that this variable physiological activity is unrelated to effectiveness. In view of the influence of plant passages on the effectiveness of Rhizobia (Allen and Baldwin) it would be interesting to determine if a corresponding change in electrophoretic potential occurs in the course of plant passages. In experiments of this nature, soil conditions must be carefully controlled in view of the fact that certain ions which affect the electrophoretic potential of bacteria may also influence infectiveness and effectiveness.

SUMMARY

1. *Rhizobium meliloti*, like other bacteria, when suspended in distilled water has a negative electrophoretic potential.
2. Variations as great as 100 per cent in the migration velocities of various strains were observed.
3. A relationship between electrophoretic potential and nitrogen-fixing ability is suggested.

4. In general, the order of the results obtained with the Northrop-Kunitz electrophoresis method was duplicated with the Falk capillary method.

5. Calcium chloride, in 1/110 M concentration, had an enormous depressing effect upon the electrophoretic migration velocity.

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Does Vitamin A Possess Vitamin D-Sparing Properties When Fed to Growing Chicks?*

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THE work described in this paper was stimulated by the work of Massengale and Nussmeier (1930), Hess and Supplee (1920), and Mussehl and Ackerson (1930) who advanced experimental evidence to show that cod liver oil is more effective in preventing nutritional legweakness in chicks than equivalent amounts of activated ergosterol, as measured by the rat method of assay for vitamin D.

Since the vitamin A content of the rickets-producing ration is ordinarily dependent on the quality and quantity of yellow corn present, it was thought that the superiority of cod liver oil for growth and ossifying potency might be due, at least in part, to the ample amount of vitamin A, which is characteristic of cod liver oil. As a result, the following experiments were conducted with the view of amplifying the legweakness producing ration with vitamin A to determine whether or not this vitamin possesses vitamin D-sparing properties.

EXPERIMENTAL

Four hundred day-old Single Comb White Leghorn chicks were divided into sixteen experimental groups of 25 birds each. The birds were housed in battery brooders and all groups received the following basal ration supplemented with various vitamin A or vitamin D carriers as indicated in Table

1. Group I received no vitamin supplement and functioned as the control group.

Basal Ration

Ground white corn	53.5%
Ground whole wheat	20.0
Wheat bran	5.0
Dried skimmilk	20.0
Sodium chloride	1.0
Calcium carbonate	0.5
	100.0

All supplements were assayed for vitamin A and vitamin D by the rat method. The vitamin D content of the alfalfa† was extremely small since 0.5 grams daily failed to protect rats against rickets while 0.015 grams of alfalfa daily was found to supply adequate vitamin A to promote growth and prevent ophthalmia in rats. The cod liver oil used was found to contain 200 A.D.M.A. rat units per gram of vitamin D and the viosterol was found to contain 50,000 units per gram. The cod liver oil had a vitamin A potency of 500 A.D.M.A. rat units per gram. Since the viosterol had a vitamin D potency equal to 250 times that of the cod liver oil, it was diluted with peanut oil so that the vitamin D content of the two sources were equal. The peanut oil was previously assayed and found to contain no vitamin A or D. All supplements were added to the basal ration at the expense of the corn and in all cases the vitamin D supplements were

† Powdered alfalfa, furnished by the California Vegetable Products Company, was used as the supplement to supply vitamin A. The viosterol used in the study was furnished by Mead, Johnson and Company and the cod liver oil was supplied by the E. L. Patch Company.

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added to the ration in equal amounts of oil so that the fat intake was comparable.

At the end of eight weeks ten representative birds in each group were removed for the determination of bone ash and line tests. Bone ash was determined by drying the tibiae, extracting them with hot alcohol and

TABLE 1. *Supplements to Basal Ration*

Group	Alfalfa (Per- cent)	Viosterol (Percent)	Cod Liver Oil (Percent)	Bone Ash of Tibiae (Percent)
I	0	0	0	—*
II	3	0	0	40.2
III	6	0	0	37.3
IV	0	.10	0	—*
V	0	.25	0	—*
VI	0	.50	0	—*
VII	3	.10	0	40.2
VIII	6	.10	0	39.9
IX	6	.25	0	38.8
X	3	.50	0	40.3
XI	6	.50	0	40.6
XII	0	0	.10	44.5
XIII	3	0	.10	44.6
XIV	6	0	.10	45.1
XV	6	0	.25	45.8
XVI	6	0	.50	48.2

* Chicks in Groups I, IV, V, and VI had all died from vitamin A deficiency before the end of the eighth week.

ether, drying and ashing. The extent of calcification was also determined by splitting the head of the bone longitudinally, immersing in a 1.5 percent solution of silver nitrate and examining the cut surface. These observations corroborated the findings obtained by the bone-ash method and for this reason are omitted from Table 1.

Table 1, in addition to giving the supplements used with the basal ration, also summarizes the data obtained. It will be noted that additions of alfalfa powder had

little or no effect on the efficiency of vitamin D so far as it can be measured by the methods employed. The superiority of cod liver oil from the standpoint of bone formation is quite evident.

These results are in agreement with the recent conclusions of Russell and Klein (1931) who used dried carrots as the source of vitamin A. In conclusion one interesting observation should be recorded, viz., that the line tests of tibiae of birds in Groups X and XI (which received the maximum amounts of viosterol with ample amounts of alfalfa) indicated that there was a tendency for ossification in the shaft at the expense of the metaphysis, where early ossification is generally most evident.

CONCLUSIONS

The addition of vitamin A, in the form of alfalfa leaf meal, to legweakness producing rations, supplemented with irradiated ergosterol, does not appear to possess a sparing effect so far as the ossifying properties of vitamin D are concerned.

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COMPOSITION OF CURRENT AND PREVIOUS SEASON'S BRANCH GROWTH IN RELATION TO VEGETATIVE AND REPRODUCTIVE RESPONSES IN *PYRUS MALUS* L.¹

WALTER THOMAS

(WITH FIVE FIGURES)

I. Introduction

The purpose of this experimental project was to determine the response of apple trees to different combinations of the principal fertilizer constituents, nitrogen, phosphorus, and potassium, but with the elimination of the variables usually present in most field experiments. The phase of the investigation reported in this paper is concerned with the association of vegetative and reproductive responses to the internal conditions as represented by analysis of metabolically active tissues at different periods in the growth cycle.

1. PRINCIPAL NUTRIENT ELEMENTS

Although, as is pointed out by LIVINGSTON (96), it is conceivable from the physiological viewpoint that conditions may be found under which any element other than the traditional ten is essential to growth, experience has shown that cultivated plants are, under ordinary field conditions when the percentage base saturation (152) is not the limiting factor, far more sensitive to changes in the nitrogen, phosphorus, and potassium content of soils than to any of the other elements. There is evidence (2, 139, 165) that the peculiar and characteristic effects of nitrogen, phosphorus, and potassium may be due to the greater motility of their ions and, in the case of potassium, to the effect on the streaming rate of protoplasm. COLLA (30), for example, finds that potassium has a greater effect in increasing the streaming rate of the protoplasm of *Chara crinita* than sodium or magnesium. But, whatever may be the mechanism of their action, the existence of the fertilizer industry is sufficient evidence of the agricultural economic importance of these three elements. From the standpoint of behavior and function, there is great need for more definite information concerning the rôle played by each of these dominant nutrient elements with respect to growth, reproduction, and other physiological functions.

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2. SEQUENCE OF DISTRIBUTION OF VARIATIONS IN CONCENTRATION OF ELEMENTS WITH RESPECT TO TIME

The experiences of the last decade (18, 46, 47, 69, 85, 86, 87, 88, 89, 90, 91, 174, 201) have confirmed and extended the value of the earlier investigations (230, 231) in which the concentration of certain elements or groups of elements was determined in the metabolically active tissues at critical periods in their vegetative development. Investigations of this type have proved of special service in the diagnosis of the nutritive conditions produced in plants by the application of *different combinations* of the principal nutrient elements. In this way we may describe metabolic processes with reference to their sequence of changes with respect to time. Although this method of approach does not necessarily enable the "causal relations" (97) to be determined but probably only the "residual effects" (antecedent influential conditions), the knowledge obtained from studies of this type supplies a valuable picture of the dynamics of physiological processes. Experiments planned in this manner have already enabled the investigator to detect quantitatively the lack of optimum (well-balanced) nutritive conditions and to prescribe remedial measures (208).

The sequence of changes with respect to the chemically reactive ions or reactive groups determined in this manner is not sensibly influenced by the range of variation in the meteorological conditions hitherto encountered in experiments with fruit trees. This is indicated by the similarity in direction of and relative magnitude of the seasonal changes of reactive groups or complexes, such as glucose, sucrose, starch, and also of the elements potassium and phosphorus in the present experiments compared with those reported under widely different meteorological conditions for the same species by others (69, 122). The writer has also obtained indications (201, 202) that the seasonal variations (*i.e.*, the time-concentration-curve) of the various nitrogen fractions throughout a vegetative cycle have also been affected primarily by internal causes.

3. MECHANISM OF FERTILIZER ACTION

The present investigation is characterized by a remarkably rapid but differential response of the plants to relatively small applications—low concentrations—(page 396) of the elements nitrogen, phosphorus, and potassium, the response varying according to the combination in which these elements are present. Marked differentiation between the trees of certain treatments was apparent even within a period of six weeks after application of the nutritive elements, and the differences in response became more intensified as the experiment progressed. How do such small quantities have such marked results? How have these trees grown so differently? Is the mechanism of fertilization one of compensation or of stimulation?

According to LIEBIG'S (94) "mineral theory," fertilizers are effective only in so far as they supply to the soil the mineral elements in which the soil is relatively deficient. In 1903 WHITNEY and CAMERON (224, 225) stirred the scientific world by the announcement that plants obtain their nutrients from the substances *already existing in the soil solution*. The theory was advanced that the soil solution was approximately constant in composition, *viz.*, a saturated solution of the minerals common to all soils, and that accordingly the soil solution was in *simple equilibrium* with the soil-forming minerals. The addition of fertilizers, therefore, according to these investigators, could not increase the concentration of the soil solution. Any response to applications of nitrogen, phosphorus, or potassium by plants was attributed by WHITNEY and CAMERON to their action on the physical properties of the soil, to their effect in neutralizing toxins, and also to their catalytic or stimulatory action. This view was vigorously disputed, especially by HOPKINS (73), and also by the Rothamsted group (56, 168) who showed the fallacies of this conception. Experimental evidence has now accumulated showing that the concentration and composition of the soil solution are continuously changing (20, 66, 177, 186, 217), being modified by several factors: (a) the amount of soil moisture, (b) the rate of absorption and utilization of elements by the plant, (c) the respiratory coefficient of the roots, and (d) the activity of the microorganisms of the soil and of the rhizosphere.

The theory of *activation* as opposed to *compensation* has recently been revived by Italian investigators, especially by MAGRINI (108). The latter concludes that fertilizers act to stimulate the plant to absorb certain other nutrients found in the soil. But as the sole evidence submitted in support of the theory is the observation that only a negligible quantity of the nitrogen of the subsoil is removed by the crop after each cultivation, this would seem to be insufficient to justify the conclusion.

4. EFFECTS OF SALTS ON THE SOIL SOLUTION

Numerous investigators (43, 51, 182, 213, 218) have shown that fertilizer salts produce highly complicated effects. They may cause marked changes in the composition of the soil solution. For example, it has been shown by the writer (197) that the addition of $\text{CaH}_4(\text{PO}_4)_2$ to the Hagerstown silty clay loam soil used in these experiments increased the Fe and Al in the soil solution, and that NaNO_3 and K_2SO_4 increased the amount of PO_4 in solution, whereas both $\text{CaH}_4(\text{PO}_4)_2$ and K_2SO_4 increased the amount of Na in solution. The effect of fertilizers, accordingly, will necessarily depend on the resultant effect on the dominant nutritive ions of the soil solution (208). The reciprocal effects of nitrogen, phosphorus, and potassium in relation to their absorption by plants has been treated in detail by the writer in another paper (208).

5. DO ROOTS DERIVE THEIR NUTRIENTS FROM A SOLUTION
MUCH MORE CONCENTRATED THAN THAT OBTAINED
BY DISPLACEMENT METHODS?

This question is of paramount importance, for upon its answer depends the validity of the interpretation of the results of experiments which have for their purpose the determination of the critical concentrations of essential ions, *i.e.*, the concentration with respect to any element or ion below which the total absorption by the plant per unit of time is inadequate.

Many plant physiologists suggest that the chemotropic property possessed by the fine roots causes the root to grow from regions of low concentration of solutes to regions of higher concentrations. These regions of high concentrations would, presumably, be produced initially by the action of the CO_2 and other acids formed in the metabolic processes of the microorganisms growing around the colloidal and mineral complex of the soil particles (103). The mechanism involved may be explained in part at least by the GIBBS-DONNAN law (34, 35, 208) and in part by the processes discussed in detail by MATTSON (116, 117, 118, 119, 120). Diffusion processes then would result in the formation of a series of concentric zones of progressively decreasing concentration of solutes. As the plant grows the carbon dioxide evolved in the respiration of the roots becomes the dominating factor in the dissolution of the soil particles (207), furthering the formation of these concentric zones of decreasing concentration. It follows that one of the fundamental problems in studies on the *interrelationship between plant and soil* is the question whether plants can or cannot absorb colloids (204), and whether the actual soil solution can be obtained by any of the numerous procedures proposed to achieve this purpose, such as water extraction (17, 19, 68, 217), pressure (95, 125, 162, 216), displacement (31, 74, 148, 171, 216), centrifugal (15, 16), and freezing-point (64) methods.

An examination of the data by BURD (17, 19), HOAGLAND (64, 67, 68), MILLAR (121), PARKER (148, 149, 153), STEWART (186), TEAKLE (195), and also by the writer (197) indicates that the soil solution obtained by displacement methods gives in the case of slightly acid or neutral soils a rather close index of the magnitude of the critical concentrations for different plants of the principal nutrient ions and of the supplying power of the soil with respect to these ions. It has frequently been observed (19, 52, 149), however, that crop growth is not related to the concentration of phosphate in the soil solution. For a critical analysis of the anomalous position of phosphorus the reader is referred to papers by BURD and MARTIN (21), GREENHILL (52), and TIDMORE (209).

It is of interest to note that the critical concentration for PO_4 may be as low as 0.20 p.p.m. in flowing cultures for corn (149, 209), and the critical

concentration for K in the case of alfalfa and clover may be under 0.5 p.p.m., although relatively higher values for some plants are reported, *e.g.*, 1 p.p.m. PO_4 for barley (67) and over 4 p.p.m. K for tomatoes (78). It must be noted, however, that TRELEASE and LIVINGSTON (212) have cautioned against the attempt to determine the comparative physiological values of different nutrient solutions without a knowledge of the climatic conditions under which the tests are made.

Specific soils, of course, vary in their ability to renew a particular nutrient in the soil solution at suitable levels of concentration, *i.e.*, above the critical concentration; and this power of renewal will depend upon the resistance to decomposition of the mineral particles and to the nature of the colloid complexes. It is necessary, too, to distinguish between the two forms of soil solution: the micellar solution, being an integral part of the micelle itself, cannot be renewed.

6. ABSORPTION OF MINERAL ELEMENTS

In earlier papers the writer (204, 205, 206, 207, 208) has discussed some of the factors determining the absorption of nutrient elements by the plant. The effect or action of a specific ion under given conditions of light and temperature as related to its absorption and utilization within the plant is dependent upon the concentration of the media, upon the nature of the other ions present, and upon the ratio of the concentrations of equally charged ions to one another.

There are a number of experiments (65, 109, 163, 196) which indicate that variations in the *total concentrations* of ions may have less effect on metabolism than changes in the *proportion* of the ions. Since continual fluctuations in the concentration and ratio of the nutritive ions in the soil solution occur, it might be expected that by the processes of adaptation plants would be gradually adjusted to somewhat wide variations in this respect; or, as HOAGLAND (67) expresses it, "there is no doubt that all kinds of interionic effects occur during absorption, varying with the composition and concentration of the culture solution, stage of growth and climate."

The soil conditions involved in an adequate understanding of the problems connected with the interrelationship of the plant and soil have been briefly sketched here in order that the reader may keep in mind the significance of the substrate in relation to the facts brought out in this paper. Only the analyses of the plant are reported; the soil conditions have been discussed in previous papers (198, 208).

II. Materials and methods

1. SOIL USED

During the period 1918-20, the late WILLIAM FREAR and the writer made several attempts to obtain information regarding the effect of the

principal fertilizer elements on the internal conditions of apple trees growing in the experimental orchards then conducted by the Pennsylvania State College throughout the State. But, inasmuch as no consistent results could be obtained, this plan was abandoned for one in which soil and also stock variations were eliminated. This was accomplished by the use of boiler-plate cylinders, 42 in number, 5 feet in diameter and 5.5 feet deep, which were sunk into the ground and filled with a virgin soil (a silty clay loam of the Hagerstown series) adjoining a strip of woodland adjacent to the college experimental orchard. The complete chemical and mineralogical analyses of this soil have been reported elsewhere (198). Six inches of crushed limestone were placed at the bottom of each cylinder to prevent capillary rise of the surrounding soil into which the cylinders were sunk. The soil used in these cylinders was removed by layers and each horizon was thoroughly mixed during a period of many months before introduction into the cylinders in October, 1921. The total weight of soil used was 210 tons. This soil was then allowed to remain fallow for one year to permit the effects of the climatic factors on the physical, chemical, and microbiological conditions to become uniform in all cylinders.

2. PLANTING

In the spring of 1922, standard stocks vegetatively propagated from a single parent secured from the East Malling Agricultural Research Station, England, whip-grafted to Stayman Winesap scions, were planted. The scions were obtained from a tree whose performance was known for two bud generations (3). Growth measurements made at the end of the years 1922, 1923, and 1924 before fertilizer applications were made showed no significant differences between the trees in adjacent rows (3) (table II).

3. FERTILIZER TREATMENT

Nitrogen, phosphorus, and potassium were supplied in the form of C.P. salts, singly and also in the various combinations shown in the diagram (fig. 1). Analysis gave NaNO_3 , 16.45 per cent. N; $\text{CaH}_4(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$, 56.30 per cent. P_2O_5 ; and K_2SO_4 , 53.40 per cent. K_2O . The following amounts per tree were added: NaNO_3 , 918 gm.; K_2SO_4 , 293 gm.; and $\text{CaH}_4(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$, 534 gm., equivalent per acre of 100 trees to 35 lb. N, 35 lb. K_2O , and 70 lb. P_2O_5 . If calculated in terms of percentage amounts present in the first 8 inches of surface soil (horizon A), the amounts added are equivalent to 0.0054 per cent. N, 0.0054 per cent. K_2O , and 0.0108 per cent. P_2O_5 , respectively. These quantities of salts were not applied at one time; the first application was made in April, 1925, and the second on May 3, 1925.

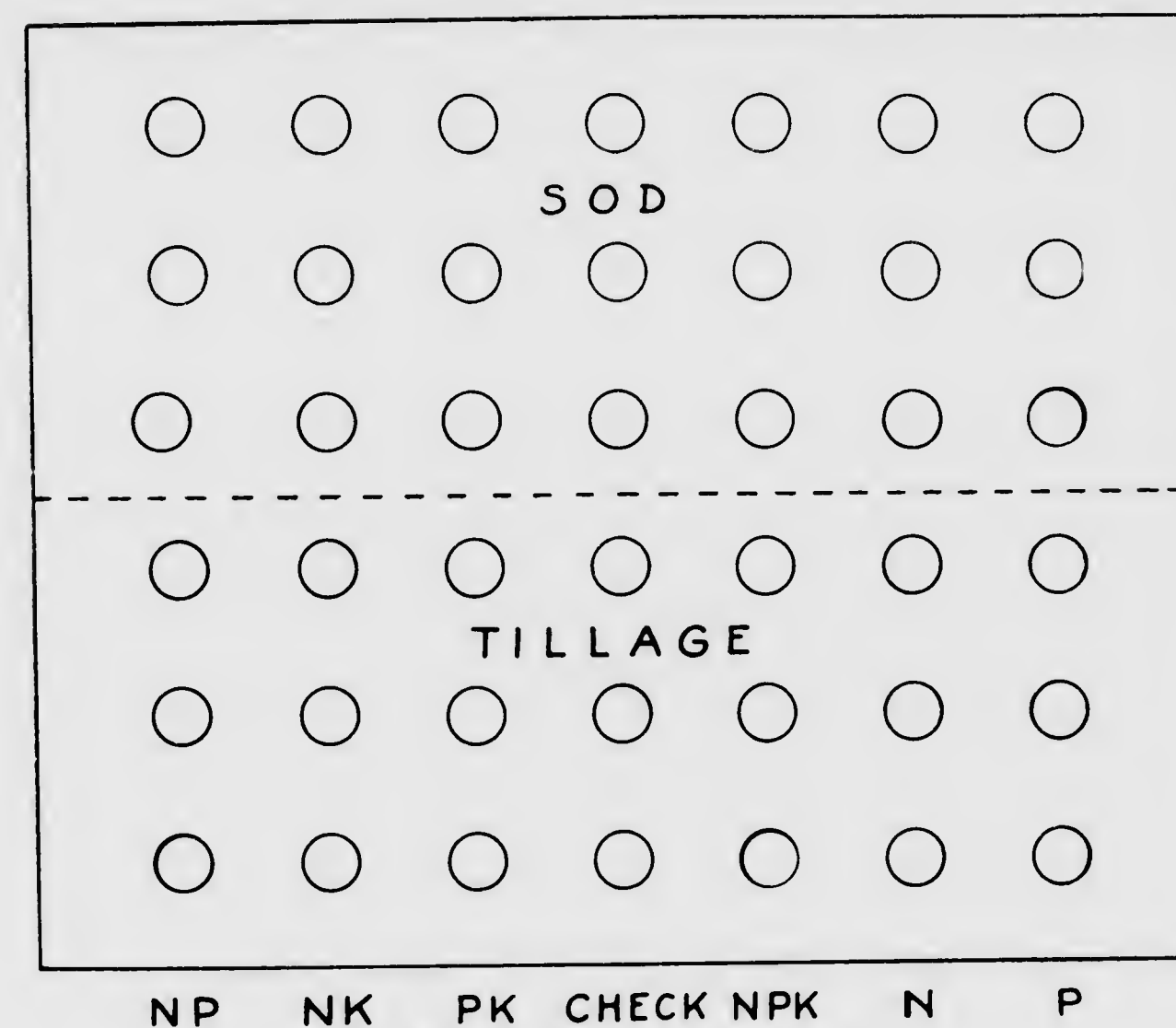


FIG. 1. Plan of experiment.

It should be noted that none of the trees bloomed before the treatment with fertilizers, *i.e.*, there were no blossoms in the spring of 1925 and, therefore, no fruit buds were formed in 1924. On May 12, 1926, the year following the fertilizer additions, the trees were deflorated, *i.e.*, the flower buds were removed just before the normal opening of flowers, to eliminate the influence of reproductive activities and fruit dominance on the growth and composition of the actively growing tissues (9, 127, 128).

4. SELECTION OF SAMPLES FOR ANALYSIS

Inasmuch as this investigation was not concerned with the dynamics of the photosynthetic processes, but rather with the relationship of the internal changes produced by the principal nutrient elements to the vegetative and reproductive responses, it was decided in the first instance to examine the organs used for transport of food and for storage. The question then was, to what extent does the composition of the current and previous season's branch growth reflect the changes in metabolism which resulted in such striking differences in the growth of the trees in this experiment?

KRAYBILL, POTTER, and WENTWORTH (84), LOOMIS (100), and also POTTER and PHILLIPS (156, 157) suggest that the samples for analytical examination should be confined preferably to new spur growth, the spurs

being nothing more than lateral branches which for some reason have made only limited growth. In the present experiment it was at the outset considered doubtful whether the spurs would provide sufficient material for the work planned. But even so there are sound reasons why investigations bearing on the internal factors associated with growth and fruit-bud formation should not be confined to the spurs alone.

A. REASONS FOR CHEMICAL EXAMINATION OF BRANCH GROWTH.—The chemical examination of the current and previous season's branch growth is of importance (201), for changes in the metabolic direction of the living cells of the woody stems must precede those in the developing bud. Hence, in the last analysis, conditions further back in the tree would *a priori* be expected to have considerable influence on the chemical system determining growth and fruiting. The investigations of HOOKER (70) and of HOOKER and BRADFORD (72), moreover, indicate that the variations in the chemical composition of bark taken from the scaffold limbs parallel closely those in the spurs, new growth, and last year's branch growth. And the writer (201, 202) has shown that, in so far as the nitrogen cycle is concerned, the seasonal variations in concentration of the various nitrogen fractions parallel one another in the current and previous season's branch growth.

B. DO INDIVIDUAL BRANCHES AND SPURS ACT AS A UNIT?—The principal difficulty in sampling fruit trees lies in obtaining homogeneous material without sensibly changing the nutritive conditions. If it could be shown that each spur acts as a unit, then errors arising from variability would cease to be much of a factor, even when only a few trees are available for analysis. Experimental and observational work exists that might seem to support the theory that individual branches and spurs act as a unit (8, 105, 166, 226). Thus, AUCHTER concluded from experiments on apple trees that mineral nutrients absorbed by the roots on one side of a plant are in large measure translocated to and used only by the trunk, limbs, and leaves directly above them; *i.e.*, there is no cross-over from one sector to another. But, as NaNO_3 was the only salt used in AUCHTER's experiments, and in view of the writer's results that nitrates are for the most part reduced in the fine roots of *Pyrus malus* (203), the possibility exists that AUCHTER's conclusions are valid only for *organic* materials (which move only in the phloem tissue) and not for the *mineral* (*i.e.*, ash) materials (29, 111, 112, 113, 114, 115). Neither the observations of HARDY (57) nor of MACK (107) support the view of the individuality of the spur. The latter found a uniformity of behavior of all spurs with respect to "off-year" bearing, "on-year" bearing, and "biennial" bearing.

These conflicting views may, perhaps, be reconciled when consideration is given to the fact that a fruit tree, although in effect a single organism, may be treated as a colony of competing units (11, 62). But even so the idea of independence can be carried too far, for a spur deprived of its own leaves can receive enough nutrient supply from adjacent parts of the tree to function actively (62). Moreover, the behavior of different varieties of fruit trees differs in this respect (62).

5. COLLECTION OF SAMPLES

On September 1, 1925, and succeeding dates (tables V to IX), samples of the current and previous season's branch growth were taken in the manner already described (200, 201) from the trees growing in the rims that were seeded to bluegrass on May 27, 1924, *i.e.*, from the trees growing in sod. In the following year (1926) samples were taken on April 4, June 21, August 15, and November 1. These periods may be characterized as follows: September 1, period of cessation of active growth; April 4, period of bud swelling; June 21–August 15, period of active growth; November 1, leaf fall and beginning of period of winter rest. As a matter of record, a summary of the meteorological conditions is appended in table I.

TABLE I
SUMMARY OF METEOROLOGICAL DATA

DATES	MEAN MAXIMUM	MEAN MINIMUM	RAINFALL	SUNSHINE	HUMIDITY
	° F.	° F.	inches	per cent.	per cent.
1925					
Aug. 15–Nov. 1 ...	67.4	48.5	5.78	81.5
1926					
Apr. 4–June 30 ...	69.2	43.4	7.34	70.4	72.5
June 30–Aug. 15 ...	83.0	61.4	5.35	61.4	80.3
Aug. 15–Nov. 1 ...	68.5	50.4	12.41	87.1

6. SEPARATION OF BARK AND WOOD

The bark (periderm, cortex, phloem) was separated from the wood (outer xylem, inner xylem) and each analyzed separately. In the present work no attempt was made to divide the complex constituents (tissues) of the bark and xylem, although it is realized that very limited regions may be involved in a particular response (211, 215).

GRADIENTS.—There undoubtedly do exist slight differences in composition between the terminal, middle, and basal portions of the bark and wood, respectively, of shoots (215). But there is no evidence as yet of either a positive or negative gradient. TUFTS (215) reported that differences in

composition between the various portions so far as location up and down the shoot is concerned are relatively insignificant, compared with those existing between bark and wood in the same portions. Preliminary work to test this point carried out by the writer prior to the present studies showed very similar results.

In the usual macroscopic method of analysis, cells of admittedly widely different nature and function are taken: epidermal, vascular, and parenchymatous tissues are mixed indiscriminately. It is, therefore, only the predominant internal relations that are revealed by the methods adopted in the present study.

7. ANALYTICAL METHODS

A. DO THE VALUES IN TOTAL CARBON DETERMINATIONS REPRESENT POTENTIALLY MOBILE AND METABOLICALLY REACTIVE COMPOUNDS?—In the present investigation, fractionation of the nitrogen compounds similar to those previously carried out by the writer (201, 202) has not been undertaken. The possibilities and limitations of nitrogen partition investigations in this species have already been clearly defined (202). These earlier studies have shown, moreover, that the respective nitrogen fractions were measurable functions of the total nitrogen, even under widely differing external environments. Thus, the total water-soluble N, the non-protein N, and the amino N closely paralleled the total N throughout the growth cycle; the amide N and "rest" N varied inversely with them. It would seem, therefore, that amino N must be connected with protein synthesis and "rest" N with protein degradation. Accordingly, since the sequences of variations (changes) in the distribution of partially synthesized proteins parallel the total N, the seasonal fluctuations of the former may be predicted from the time-concentration curves of the latter.

On the other hand, if any definite relationships exist between the total carbohydrate and its various fractions, they have not been identified in any metabolism studies thus far carried out. It was considered desirable, therefore, to undertake the determination of such carbohydrate fractions as are amenable to present methods of analysis rather than the determination of total carbon preferred by some investigators (63), who consider that the determination of total carbon is sufficient to establish the relationship existing between the carbon and nitrogen compounds present. HICKS (63) justifies the determination of total carbon, without fractionation according to the usual methods, on the basis that the primary value of the C/N relation lies in the fact that the growth of a plant is dependent upon the balance between the metabolic processes of carbon assimilation and respiration on the one hand and absorption of nitrogen from the soil on the other; and that, since both processes involve the use of "raw" inorganic material in the

form of carbon dioxide and nitrates, the relation of real importance in the plant itself, according to her view, is that of elemental carbon to elemental nitrogen. Although this investigator recognizes the fact that part of the assimilated carbon is transformed into immobile compounds, such as lignin, she maintains that these immobile compounds have not been shown to lie outside the vital system, and, therefore, to be without influence on the plant. It would appear that the problem of the plant physiologist is not so simple in this respect as that of the animal physiologist (81). Nevertheless, *the burden of proof remains with the proponents of the "total" carbon idea to show that the carbon compounds of plants are as reactive and mobile as the nitrogen compounds with which they are compared.*

B. WATER.—(1) The imbibitional water was determined by drying the material in a drying oven at a temperature of 70°–80°. The samples were afterwards left exposed to the air for several weeks and weighed when equilibrium had been reached. (2) The hygroscopic water was determined by heating 2 grams of the finely ground materials *in vacuo* at 100°.

C. ASH.—Ten grams of the finely ground material were ignited in an electric muffle furnace at a temperature not exceeding 450° to avoid loss of potassium salts.

D. POTASSIUM.—The usual LINDO-GLADDEN method was employed (7).

E. PHOSPHORUS.—The phosphorus was precipitated as the ammonium phospho-molybdate after the method of STEWART (186) and the precipitate was titrated immediately according to RICHARDS and GODDEN's modification (164) of the PEMBERTON-NEUMANN method (133, 151).

F. NITROGEN.—Nitrogen was determined by the usual KJELDAHL method (7). Only traces of nitrates are present in apple tissues (38, 203).

G. CARBOHYDRATES.—The estimation of the initial separation of carbohydrates was effected by the method proposed by the Committee on Methods of Chemical Analysis of the American Society of Plant Physiologists into a "soluble" and an "insoluble" fraction (5). In the "soluble" portion: (1) reducing sugars (glucose, fructose, pentose, arabinose, and xylose) and (2) non-reducing sugars (sucrose and other disaccharides) were determined. Glucosides such as phloridzin (61) were not separately estimated; these compounds would be included in the latter group.

It is of interest to note that it has recently been questioned if phloridzin is a true glucoside. MOELWYN-HUGHES (123) finds that phloridzin is not hydrolyzed by emulsin but by another enzyme occurring freely in nature—probably β -(γ)-fructosidase. Moreover, he also points out that all the published data on the velocity of hydrolysis of glucosides by acids indicate that phloridzin resembles, with respect to velocity coefficient and critical increment for hydrolysis, the γ -fructosides (sucrose, raffinose, melezitose) more closely than the normal glucosides (salicin, arbutin, maltose, etc.).

The determinations of the various fractions were made according to the writer's method (199). The acid-hydrolyzable "insoluble" fractions (4, 5) include the ill-defined groups designated by SCHULZE (172) as "hemicellulose," and which may function as reserve material (129, 167). In the present investigation the term includes hexosans, pentosans, and such mixed polymers as pectins and gums.

III. Presentation of results

1. GROWTH MEASUREMENTS

Throughout the period 1922-24 inclusive, *i.e.*, before the nutrient salts were added, there was no significant difference in growth measurements in trees from different rows (3) (table III). The response of the trees to ni-

TABLE II
EFFECT OF TREATMENTS ON GROWTH OF GRASS CUT IN 1925

TREATMENT	WEIGHT OF GRASS CLIPPINGS
	<i>gm.</i>
Check	845
NPK	5708
NP	5365
NK	2673
PK	1760
N	3743
P	1457

trogen additions was especially striking. Within six weeks after the first application in the spring of 1925, the leaves of the trees receiving nitrogen additions changed from a pale green to a deep green color. At the end of

TABLE III
BRANCH ELONGATION IN CENTIMETERS OF SEASON'S BRANCH GROWTH (1922-1926)

TREATMENT (AFTER 1924)	Sod				
	1922	1923	1924	1925*	1926
Check	851	3270	5005	3051	985
NPK	863	2560	4662	6991	6578
NP	429	2637	4983	6475	7795
NK	648	3402	5094	5808	5031
PK	762	2517	4881	3247	2073
N	570	2796	3844	5564	5243
P	635	2696	4537	3247	1790

* First year of fertilizer application.

this period marked differences in growth responses were observed. The soil condition with respect to supply of nutrients at this time is indicated by the combined weight of the grass clippings (table II), taken in June, 1925, from the "sod" cylinders and also of the combined branch elongation at the end of the 1925 growing season (3) (table III) for all three trees in each treatment.

The effect of the differential fertilizer treatments given in the spring of 1925 on the fruit bud formation of that year is shown in table IV, which gives the number of blossom clusters removed from the three trees in each treatment in 1926 (3) when the trees were deflorated.



FIG. 2. Typical tree growing in sod treated with the three nutrient salts: NaNO_3 , K_2SO_4 , and $\text{CaH}_4(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$.

TABLE IV

EFFECT OF TREATMENTS ON NUMBER OF BLOSSOM CLUSTERS IN 1926

TREATMENT	SOD	CULTIVATED
Check	0	6
NPK	328	353
NP	145	334
NK	102	17
PK	0	28
N	21	71
P	0	13

It may be pointed out here that the tips of the shoots of the check trees and also of those receiving only phosphorus had relatively little active cambium compared with the trees of other treatments. The nitrogen-treated trees were differentiated from the others in having less xylem with thinner walls than the trees without nitrogen additions. The cambium, too, of the trees receiving added nitrogen was active even to the base of the stem.

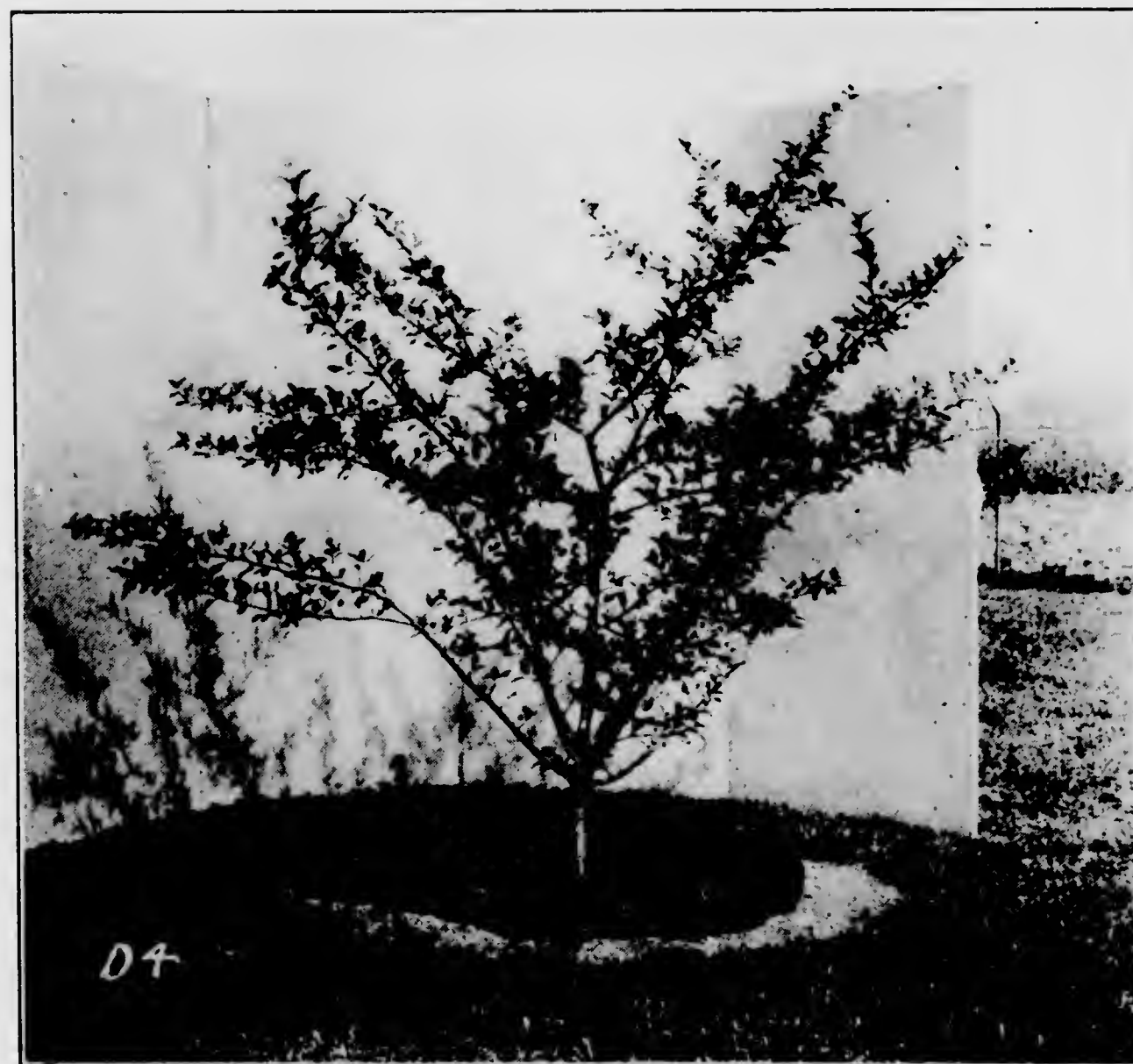


FIG. 3. Typical tree growing in sod without nutrient salt additions.

Figure 2 shows a representative tree from the row in sod and receiving all three of the nutritive elements nitrogen, phosphorus, and potassium. Figure 3 shows a representative check tree receiving no nutrient additions.

2. ANALYTICAL DATA

In the following tables the term "glucose" will be used to designate the reducing sugars estimated as glucose (p. 401), and the term "sucrose" to indicate the non-reducing sugars and glucosides (p. 401). The analytical results are presented in tables V-IX.

TABLE V

PERCENTAGES OF WATER IN SEASON'S AND IN ONE-YEAR BRANCH GROWTH (WOOD AND BARK SEPARATED)

LABORATORY NO.		SAMPLING DATE	TREATMENT	IMBIBITIONAL WATER		HYGROSCOPIC WATER		TOTAL WATER	
WOOD	BARK			WOOD	BARK	WOOD	BARK	WOOD	BARK
7	8	Sept. 1, 1925	Check	39.42	47.00	2.23	2.72	41.65	49.72
58	59	Apr. 4, 1926		42.08	51.60	2.06	2.80	44.14	54.40
89A	89B	June 30, "		40.70	50.16	1.80	2.55	42.50	52.71
98	99	Aug. 15, "		38.80	46.70	1.40	2.70	40.20	49.40
185	186	Nov. 1, "	NPK	37.24	45.05	1.46	2.30	38.70	47.35
9	10	Sept. 1, 1925		43.11	53.70	2.54	4.05	45.65	57.75
50	51	Apr. 4, 1926		45.68	55.60	2.72	3.96	48.40	59.56
90A	90B	June 30, "		45.93	54.60	2.32	4.20	48.25	58.80
94	95	Aug. 15, "	NP	44.10	52.90	2.40	4.80	46.58	57.70
182	183	Nov. 1, "		40.60	48.70	2.80	3.85	43.40	52.55
1	2	Sept. 1, 1925		43.97	50.60	2.75	3.85	46.72	54.45
82	83	Apr. 4, 1926		44.25	55.00	2.72	3.57	46.97	58.57
85A	85B	June 30, "	NK	45.15	52.30	2.35	3.90	47.50	56.20
110	111	Aug. 15, "		44.43	50.10	2.10	3.80	46.53	53.90
194	195	Nov. 1, "		38.70	47.60	2.02	4.00	40.72	51.60
3	4	Sept. 1, 1925		42.50	50.12	2.60	3.60	45.10	53.72
74	75	Apr. 4, 1926	PK	44.36	54.80	2.52	3.30	46.88	58.10
86A	86B	June 30, "		44.22	51.45	2.38	3.75	46.60	55.20
106	107	Aug. 15, "		43.07	48.20	2.15	3.80	45.22	52.06
191	192	Nov. 1, "		38.04	50.50	2.06	3.90	44.10	50.30
5	6	Sept. 1, 1925	N	43.38	50.27	2.50	3.56	45.88	53.83
66	67	Apr. 4, 1926		44.26	54.70	2.08	3.76	46.34	58.46
88A	88B	June 30, "		43.10	50.86	2.30	3.85	45.50	54.71
102	103	Aug. 15, "		41.60	49.30	2.60	4.00	44.20	53.30
188	189	Nov. 1, "	P	40.65	49.40	2.35	3.50	43.00	52.90
11	12	Sept. 1, 1925		42.10	52.20	2.40	4.00	44.50	56.20
42	43	Apr. 4, 1926		42.50	55.40	2.78	3.57	45.28	58.97
91A	91B	June 30, "		44.08	54.05	2.15	4.15	46.23	58.20
95	96	Aug. 15, "	P	42.20	51.73	2.30	3.90	44.50	55.63
179	180	Nov. 1, "		40.50	47.80	2.25	4.00	42.75	51.80
13	14	Sept. 1, 1925		38.95	47.13	2.25	2.72	41.20	49.85
34	35	Apr. 4, 1926		42.38	51.10	2.20	2.65	44.58	53.75
92A	92B	June 30, "	P	41.28	50.18	1.72	2.40	43.00	52.58
97	98	Aug. 15, "		38.40	47.10	2.00	2.80	40.40	49.90
176	177	Nov. 1, "		37.55	46.45	1.65	2.65	39.20	49.10

TABLE VI

AMOUNTS OF GLUCOSE, SUCROSE, TOTAL SUGARS, STARCH, HEMICELLULOSE, AND "TOTAL" AVAILABLE CARBOHYDRATES AS PERCENTAGES OF FRESH AND DRY WEIGHTS, RESPECTIVELY, OF THE SEASON'S AND ONE-YEAR BRANCH GROWTH (WOOD AND BARK SEPARATED)

LABORATORY NO.		SAMPLING DATE	TREAT- MENT	PERCENTAGES OF FRESH WEIGHT OF MATERIAL											
				GLUCOSE		SUCROSE		TOTAL SUGARS		STARCH		HEMICELLULOSE		TOTAL AVAIL- ABLE CARBO- HYDRATES	
				WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK
7	8	Sept. 1, 1925	Check	0.117	0.292	0.140	0.503	0.257	0.794	5.946	5.742	18.042	10.483	24.244	17.019
58	59	Apr. 4, 1926		0.229	0.401	0.078	0.310	0.307	0.812	6.173	5.016	16.021	9.758	22.500	15.586
89A	89B	June 30, "		0.104	0.189	0.035	0.355	0.138	0.544	4.859	5.490	17.290	10.427	22.287	16.462
98	99	Aug. 15, "		0.167	0.278	0.114	0.430	0.281	0.708	5.532	6.527	18.299	11.132	24.111	18.368
185	186	Nov. 1, "		0.337	0.400	0.153	0.316	0.490	0.716	6.743	6.349	17.783	11.583	25.017	18.649
9	10	Sept. 1, 1925	NPK	0.217	1.014	0.228	0.668	0.446	1.682	4.033	3.046	13.756	7.977	18.234	12.705
50	51	Apr. 4, 1926		0.325	0.768	0.175	0.384	0.501	1.153	4.051	1.415	11.796	7.869	16.347	10.439
90A	90B	June 30, "		0.181	0.692	0.202	0.124	0.383	0.816	1.656	2.493	11.519	8.446	13.559	11.754
94	95	Aug. 15, "		0.241	0.973	0.257	0.275	0.498	1.248	2.408	3.215	13.857	8.058	16.227	12.521
182	183	Nov. 1, "		0.404	1.205	0.255	0.664	0.719	1.869	4.415	2.159	13.324	10.425	18.457	14.453
1	2	Sept. 1, 1925	NP	0.234	1.098	0.202	0.706	0.437	1.804	4.529	3.553	12.749	8.126	17.716	13.483
82	83	Apr. 4, 1926		0.286	0.766	0.159	0.423	0.445	1.189	4.667	1.595	11.858	8.526	16.969	11.310
85A	85B	June 30, "		0.210	0.701	0.184	0.197	0.394	0.898	1.890	2.602	12.731	8.961	14.700	12.461
110	111	Aug. 15, "		0.257	1.014	0.267	0.401	0.524	1.415	2.940	3.467	12.908	8.759	16.383	13.650
194	195	Nov. 1, "		0.462	1.210	0.308	0.750	0.178	1.960	5.068	2.688	12.751	10.096	18.390	14.743
3	4	Sept. 1, 1925	NK	0.220	1.110	0.192	0.671	0.412	1.781	4.914	4.128	13.516	8.502	18.842	14.412
74	75	Apr. 4, 1926		0.297	0.859	0.133	0.453	0.430	1.311	4.249	2.263	12.499	8.988	17.179	12.562
86A	86B	June 30, "		0.203	0.717	0.235	0.215	0.438	0.932	2.429	3.069	11.983	10.022	14.851	14.022
106	107	Aug. 15, "		0.301	0.791	0.247	0.431	0.548	1.222	3.424	3.452	12.489	10.810	16.461	14.526
191	192	Nov. 1, "		0.425	1.093	0.280	0.820	0.704	1.913	3.913	2.922	13.382	11.814	17.999	15.656
5	6	Sept. 1, 1925	PK	0.162	0.254	0.179	0.323	0.341	0.577	4.654	4.178	15.522	10.125	20.517	14.881
66	67	Apr. 4, 1926		0.279	0.415	0.145	0.291	0.424	0.706	4.937	3.431	15.339	9.329	18.711	13.459
88A	88B	June 30, "		0.137	0.453	0.087	0.272	0.224	0.725	4.368	4.022	13.224	10.426	17.816	15.172
102	103	Aug. 15, "		0.206	0.318	0.156	0.294	0.363	0.612	4.788	4.483	15.507	10.839	20.657	15.934
188	189	Nov. 1, "		0.371	0.471	0.199	0.471	0.570	0.942	5.529	4.823	13.999	10.367	20.098	16.132
11	12	Sept. 1, 1925	N	0.244	0.876	0.194	0.701	0.438	1.577	4.190	4.117	15.351	8.725	19.980	14.419
42	43	Apr. 4, 1926		0.317	0.698	0.148	0.492	0.465	1.190	4.514	2.975	12.870	7.898	17.849	12.063
91A	91B	June 30, "		0.161	0.640	0.231	0.418	0.393	1.058	2.581	2.926	12.754	8.819	15.728	12.803
95	96	Aug. 15, "		0.233	0.799	0.250	0.541	0.483	1.340	3.441	3.727	14.114	9.096	18.038	14.163
179	180	Nov. 1, "		0.451	0.988	0.315	0.819	0.767	1.808	4.866	4.299	14.232	9.568	19.866	15.675
13	14	Sept. 1, 1925	P	0.129	0.376	0.112	0.707	0.241	1.083	5.733	5.667	17.910	10.105	23.885	16.855
34	35	Apr. 4, 1926		0.211	0.347	0.155	0.324	0.366	0.671	5.658	5.134	15.396	9.689	21.419	15.494
92A	92B	June 30, "		0.086	0.190	0.114	0.488	0.199	0.678	4.138	4.837	16.781	10.243	21.119	15.758
97	98	Aug. 15, "		0.161	0.301	0.131	0.326	0.292	0.651	4.765	5.882	18.363	10.341	23.423	17.149
176	177	Nov. 1, "		0.334	0.556	0.134	0.355	0.468	0.631	6.749	6.067	16.768	10.738	23.925	17.316

TABLE VI—(Continued)

LABORATORY NO.		SAMPLING DATE	TREAT- MENT	PERCENTAGES OF DRY WEIGHT OF MATERIAL											
				GLUCOSE		SUCROSE		TOTAL SUGARS		STARCH		HEMICELLULOSE		TOTAL AVAIL- ABLE CARBO- HYDRATES	
				WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK
7	8	Sept. 1, 1925	Check	0.20	0.58	0.24	1.00	0.44	1.58	10.19	11.42	30.92	20.85	41.55	33.85
58	59	Apr. 4, 1926		0.41	0.88	0.14	0.90	0.55	1.78	11.05	11.00	28.68	21.40	40.28	34.18
89A	89B	June 30, "		0.18	0.40	0.06	0.75	0.24	1.15	8.45	11.61	30.07	22.05	38.76	34.81
98	99	Aug. 15, "		0.28	0.55	0.19	0.85	0.47	1.40	9.25	12.90	30.60	22.00	40.32	36.30
185	186	Nov. 1, "		0.55	0.76	0.25	0.60	0.80	1.36	11.00	12.06	29.01	22.00	40.81	35.42
9	10	Sept. 1, 1925	NPK	0.40	2.40	0.42	1.58	0.82	3.98	7.42	7.21	25.31	18.88	33.55	30.07
50	51	Apr. 4, 1926		0.63	1.90	0.34	0.95	0.97	2.85	7.85	3.50	22.86	19.46	31.68	25.81
90A	90B	June 30, "		0.35	1.68	0.39	0.74	0.74	1.98	3.20	6.05	22.26	20.50	26.20	28.63
94	95	Aug. 15, "		0.45	2.30	0.48	0.65	0.93	2.95	4.50	7.60	25.90	19.05	30.33	29.60
182	183	Nov. 1, "		0.82	2.54	0.45	1.40	1.27	3.94	7.80	4.55	23.54	21.97	32.61	30.46
1	2	Sept. 1, 1925	NP	0.44	2.41	0.38	1.55	0.82	3.96	8.50	7.80	23.93	17.84	33.25	29.60
82	83	Apr. 4, 1926		0.54	1.85	0.30	1.02	0.84	2.87	8.80	3.85	22.36	20.58	32.00	27.30
85A	85B	June 30, "		0.40	1.60	0.35	0.45	0.75	2.05	3.60	5.94	24.25	20.46	28.00	28.45
110	111	Aug. 15, "		0.48	2.20	0.50	0.87	0.98	3.07	5.50	7.52	24.14	19.00	30.64	29.61
194	195	Nov. 1, "		0.78	2.50	0.52	1.55	1.30	4.05	8.55	5.55	21.51	20.86	31.36	30.46
3	4	Sept. 1, 1925	NK	0.40	2.40	0.35	1.45	0.75	3.85	8.95	8.92	24.62	18.37	34.32	31.14
74	75	Apr. 4, 1926		0.56	2.05	0.25	1.08	0.81	3.13	8.00	5.40	23.53	21.45	32.34	29.98
86A	86B	June 30, "		0.38	1.60	0.44	0.48	0.82	2.08	4.55	6.85	22.44	22.37	27.81	31.30
106	107	Aug. 15, "		0.55	1.65	0.45	0.90	1.00	2.55	6.25	7.20	22.80	22.55	30.05	30.30
191	192	Nov. 1, "		0.76	2.20	0.50	1.65	1.26	3.85	7.00	5.88	23.94	23.77	32.20	31.50
5	6	Sept. 1, 1925	PK	0.30	0.55	0.33	0.70	0.63	1.25	8.60	9.05	28.68	21.93	37.91	32.23
66	67	Apr. 4, 1926		0.52	1.00	0.27	0.70	0.79	1.70	9.20	8.26	24.86	22.46	34.85	32.40
88A	88B	June 30, "		0.25	1.00	0.16	0.60	0.41	1.60	8.00	8.88	24.22	23.02	32.63	33.50
102	103	Aug. 15, "		0.37	0.68	0.28	0.63	0.65	1.31	8.58	9.60	27.79	23.21	37.02	34.12
188	189	Nov. 1, "		0.65	1.00	0.35	1.00	1.00	2.00	9.70	10.24	24.56	22.01	35.26	34.25
11	12	Sept. 1, 1925	N	0.44	2.00	0.35	1.60	0.79	3.60	7.55	9.40	27.66	19.92	36.00	32.92
42	43	Apr. 4, 1926		0.58	1.70	0.27	1.20	0.85	2.90	8.25	7.25	23.52	19.25	32.62	29.40
91A	91B	June 30, "		0.30	1.53	0.43	1.00	0.73	2.53	4.80	7.00	23.72	21.10	29.25	30.63
95	96	Aug. 15, "		0.42	1.80	0.45	1.22	0.87	3.02	6.20	8.40	25.43	20.50	32.50	31.92
179	180	Nov. 1, "		0.79	2.05	0.55	1.70	1.34	3.75	8.50	8.02	24.86	19.85	34.70	32.52
13	14	Sept. 1, 1925	P	0.22	0.75	0.19	1.41	0.41	2.16	9.75	11.30	30.46	20.15	40.62	33.61
34	35	Apr. 4, 1926		0.38	0.75	0.28	0.70	0.66	1.45	10.21	11.10	27.78	20.95	38.65	33.50
92A	92B	June 30, "		0.15	0.40	0.20	1.03	0.35	1.43	7.26	10.20	29.44	21.60	37.05	33.23
97	98	Aug. 15, "		0.27	0.60	0.22	0.65	0.49	1.30	8.00	11.74	30.81	20.64	39.30	34.22
176	177	Nov. 1, "		0.55	0.70	0.22	0.50	0.77	1.20	11.10	11.92	27.48	20.90	39.35	34.02

TABLE VII
AMOUNT OF ASH, NITROGEN, K₂O, AND P₂O₅ IN FRESH AND DRY WEIGHTS, RESPECTIVELY, OF THE SEASON'S AND ONE-YEAR BRANCH GROWTH
(WOOD AND BARK SEPARATED)

LABORATORY NO.		SAMPLING DATE	TREATMENT	PERCENTAGES OF FRESH WEIGHT OF MATERIAL				P ₂ O ₅			
				ASH		NITROGEN		K ₂ O		WOOD	
WOOD	BARK			WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK
7	8	Sept. 1, 1925	Check	0.957	3.469	0.088	0.327	0.108	0.346	0.118	0.129
58	59	Apr. 4, 1926		0.754	2.868	0.152	0.424	0.106	0.319	0.103	0.091
89A	89B	June 30, "		0.661	2.781	0.166	0.364	0.129	0.369	0.079	0.097
98	99	Aug. 15, "		0.927	3.087	0.111	0.364	0.119	0.379	0.102	0.116
185	186	Nov. 1, "		1.017	3.596	0.155	0.432	0.116	0.411	0.106	0.111
9	10	Sept. 1, 1925	NPK	0.935	3.207	0.261	0.393	0.136	0.404	0.136	0.129
50	51	Apr. 4, 1926		0.877	2.588	0.397	0.582	0.137	0.549	0.116	0.104
90A	90B	June 30, "		0.828	2.472	0.422	0.478	0.145	0.476	0.099	0.108
94	95	Aug. 15, "		0.899	2.939	0.321	0.474	0.134	0.431	0.128	0.149
182	183	Nov. 1, "		1.030	3.748	0.351	0.702	0.169	0.607	0.167	0.185
1	2	Sept. 1, 1925	NP	0.938	3.316	0.267	0.409	0.109	0.323	0.132	0.132
82	83	Apr. 4, 1926		0.875	2.631	0.379	0.539	0.125	0.348	0.117	0.104
85A	85B	June 30, "		0.840	2.694	0.409	0.517	0.123	0.398	0.092	0.110
110	111	Aug. 15, "		0.866	2.914	0.353	0.507	0.134	0.419	0.126	0.161
194	195	Nov. 1, "		1.186	3.520	0.412	0.600	0.169	0.465	0.178	0.186
3	4	Sept. 1, 1925	NK	0.961	3.332	0.253	0.213	0.118	0.421	0.109	0.123
74	75	Apr. 4, 1926		0.797	2.614	0.339	0.267	0.119	0.417	0.099	0.092
86A	86B	June 30, "		0.812	2.710	0.350	0.294	0.149	0.576	0.078	0.101
106	107	Aug. 15, "		0.887	2.968	0.264	0.231	0.131	0.489	0.108	0.127
191	192	Nov. 1, "		0.950	3.648	0.313	0.278	0.162	0.571	0.113	0.142
5	6	Sept. 1, 1925	PK	0.963	3.232	0.203	0.360	0.124	0.332	0.128	0.132
66	67	Apr. 4, 1926		0.789	2.575	0.280	0.407	0.131	0.353	0.119	0.102
88A	88B	June 30, "		0.699	2.649	0.290	0.403	0.145	0.451	0.079	0.112
102	103	Aug. 15, "		0.809	2.956	0.246	0.364	0.106	0.411	0.103	0.124
188	189	Nov. 1, "		0.912	3.249	0.286	0.419	0.145	0.436	0.117	0.134
11	12	Sept. 1, 1925	N	0.932	3.053	0.244	0.394	0.126	0.311	0.126	0.125
42	43	Apr. 4, 1926		0.821	2.585	0.375	0.501	0.120	0.304	0.111	0.099
91A	91B	June 30, "		0.758	2.541	0.409	0.422	0.128	0.378	0.076	0.100
95	96	Aug. 15, "		0.888	2.817	0.297	0.430	0.146	0.346	0.111	0.106
179	180	Nov. 1, "		0.967	3.441	0.313	0.511	0.146	0.395	0.119	0.125
13	14	Sept. 1, 1925	P	0.929	3.480	0.106	0.376	0.106	0.341	0.126	0.130
34	35	Apr. 4, 1926		0.798	2.775	0.202	0.402	0.102	0.333	0.116	0.093
92A	92B	June 30, "		0.684	2.802	0.205	0.379	0.107	0.382	0.083	0.099
97	98	Aug. 15, "		0.924	3.131	0.163	0.376	0.106	0.371	0.113	0.116
176	177	Nov. 1, "		1.046	3.507	0.182	0.407	0.106	0.412	0.131	0.109

TABLE VII—(Continued)
AMOUNT OF ASH, NITROGEN, K₂O, AND P₂O₅ IN FRESH AND DRY WEIGHTS, RESPECTIVELY, OF THE SEASON'S AND ONE-YEAR BRANCH GROWTH
(WOOD AND BARK SEPARATED)

LABORATORY NO.		SAMPLING DATE	TREATMENT	PERCENTAGES OF DRY WEIGHT OF MATERIAL				P ₂ O ₅			
				ASH		NITROGEN		K ₂ O		WOOD	
WOOD	BARK			WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK
7	8	Sept. 1, 1925	Check	1.64	6.90	0.151	0.65	0.185	0.689	0.203	0.256
58	59	Apr. 4, 1926		1.35	6.29	0.273	0.93	0.190	0.700	0.185	0.200
89A	89B	June 30, "		1.15	5.88	0.288	0.77	0.225	0.780	0.138	0.205
98	99	Aug. 15, "		1.55	6.10	0.185	0.72	0.200	0.750	0.170	0.230
185	186	Nov. 1, "		1.66	6.83	0.253	0.82	0.190	0.780	0.173	0.210
9	10	Sept. 1, 1925	NPK	1.72	7.59	0.480	0.93	0.250	0.957	0.250	0.306
50	51	Apr. 4, 1926		1.70	6.40	0.770	1.44	0.265	1.020	0.225	0.258
90A	90B	June 30, "		1.60	6.00	0.815	1.16	0.280	1.360	0.191	0.262
94	95	Aug. 15, "		1.68	6.95	0.600	1.12	0.250	1.155	0.240	0.354
182	183	Nov. 1, "		1.82	7.90	0.620	1.48	0.300	1.280	0.295	0.390
1	2	Sept. 1, 1925	NP	1.76	7.28	0.502	0.90	0.205	0.710	0.248	0.290
82	83	Apr. 4, 1926		1.65	6.35	0.715	1.30	0.235	0.840	0.250	0.250
85A	85B	June 30, "		1.60	6.15	0.780	1.18	0.235	0.910	0.175	0.252
110	111	Aug. 15, "		1.62	6.32	0.660	1.10	0.250	0.910	0.235	0.350
194	195	Nov. 1, "		2.00	7.48	0.695	1.24	0.285	0.960	0.300	0.385
3	4	Sept. 1, 1925	NK	1.75	7.20	0.460	0.89	0.215	0.910	0.200	0.265
74	75	Apr. 4, 1926		1.50	6.24	0.638	1.29	0.225	0.995	0.188	0.220
86A	86B	June 30, "		1.52	6.05	0.656	1.12	0.280	1.285	0.146	0.225
106	107	Aug. 15, "		1.62	6.15	0.482	1.08	0.240	1.022	0.198	0.265
191	192	Nov. 1, "		1.70	7.34	0.560	1.22	0.290	1.150	0.203	0.285
5	6	Sept. 1, 1925	PK	1.78	7.00	0.375	0.78	0.230	0.720	0.237	0.285
66	67	Apr. 4, 1926		1.47	6.20	0.522	0.98	0.245	0.850	0.222	0.245
88A	88B	June 30, "		1.28	5.85	0.532	0.89	0.265	0.995	0.144	0.242
102	103	Aug. 15, "		1.45	6.33	0.440	0.78	0.260	0.880	0.185	0.266
188	189	Nov. 1, "		1.60	6.90	0.502	0.89	0.275	0.965	0.206	0.285
11	12	Sept. 1, 1925	N	1.68	6.97	0.440	0.90	0.220	0.710	0.227	0.285
42	43	Apr. 4, 1926		1.50	6.30	0.685	1.22	0.220	0.740	0.202	0.241
91A	91B	June 30, "		1.41	6.08	0.762	1.01	0.230	0.905	0.141	0.240
95	96	Aug. 15, "		1.60	6.35	0.535	0.97	0.230	0.780	0.200	0.240
179	180	Nov. 1, "		1.69	7.14	0.546	1.06	0.255	0.820	0.208	0.260
13	14	Sept. 1, 1925	P	1.58	6.94	0.180	0.75	0.185	0.680	0.215	0.260
34	35	Apr. 4, 1926		1.44	6.00	0.365	0.87	0.185	0.720	0.210	0.200
92A	92B	June 30, "		1.20	5.91	0.360	0.80	0.180	0.805	0.145	0.210
97	98	Aug. 15, "		1.55	6.25	0.273	0.75	0.180	0.740	0.190	0.232
176	177	Nov. 1, "		1.72	6.89	0.300	0.80	0.175	0.810	0.216	0.215

TABLE VIII
RATIOS OF CARBOHYDRATE FRACTIONS, AND ALSO OF PHOSPHORIC ACID AND POTASH TO NITROGEN, CALCULATED AS PERCENTAGES OF DRY WEIGHT OF MATERIAL

LABORATORY NO.	SAMPLING DATE		TREAT- MENT	GLUCOSE/N		SUCROSE/N		STARCH/N		AVAILABLE CAR- BOHYDRATES/N		N/P ₂ O ₅		K ₂ O/N	
	WOOD	BARK		WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK
7	Sept. 1, 1925		Check	1.33	0.89	1.59	1.54	67.48	17.57	275.16	52.0	0.74	2.54	1.225	1.060
58	Apr. 4, 1926			1.50	0.95	0.51	0.97	40.48	11.83	147.55	35.8	2.48	4.65	0.696	0.753
89A	June 30, "			0.63	0.52	0.21	0.97	29.34	15.06	134.58	45.2	2.09	3.76	0.781	1.013
98	Aug. 15, "			1.51	0.76	1.03	1.18	50.00	17.79	217.95	48.9	1.09	3.15	1.081	1.034
185	Nov. 1, "			2.17	0.93	0.99	0.73	43.48	14.68	161.30	43.1	1.46	3.90	0.751	0.951
9	Sept. 1, 1925		NPK	0.83	2.58	0.88	1.69	15.46	7.75	69.89	31.6	0.94	3.04	0.521	1.029
50	Apr. 4, 1926			0.82	1.32	0.44	0.68	10.19	2.43	41.14	18.6	2.64	5.58	0.344	0.708
90A	June 30, "			0.43	1.45	0.48	0.26	3.63	5.22	32.15	24.0	3.48	4.49	0.343	1.172
94	Aug. 15, "			0.75	2.05	0.80	0.58	7.50	6.79	52.22	26.4	1.50	3.16	0.417	1.031
182	Nov. 1, "			1.32	1.72	0.73	0.95	12.08	3.07	52.59	20.5	1.30	3.79	0.484	0.865
1	Sept. 1, 1925		NP	0.88	2.68	0.76	1.72	16.93	8.67	66.23	32.9	2.02	3.10	0.408	0.789
82	Apr. 4, 1926			0.76	1.42	0.42	0.78	12.31	2.96	44.76	21.0	3.25	5.00	0.329	0.569
85B	June 30, "			0.51	1.36	0.45	0.38	3.85	5.63	35.90	24.1	4.46	4.14	0.301	0.784
110	Aug. 15, "			0.73	2.00	0.76	0.79	8.33	6.84	46.42	26.9	2.81	3.10	0.333	0.709
194	Nov. 1, "			1.12	2.02	0.75	1.17	12.30	4.48	45.12	24.5	2.32	3.22	0.410	0.661
3	Sept. 1, 1925		NK	0.87	2.70	0.76	1.63	19.46	9.91	74.61	34.5	2.30	3.40	0.576	1.011
74	Apr. 4, 1926			0.88	1.59	0.39	0.84	12.54	4.19	50.69	23.2	3.39	5.86	0.446	0.771
86A	June 30, "			0.58	1.43	0.67	0.43	6.94	6.12	42.39	27.9	4.49	4.98	0.426	1.147
106	Aug. 15, "			1.14	1.53	0.93	0.83	12.96	6.67	62.34	29.9	2.43	4.08	0.496	0.946
191	Nov. 1, "			1.36	1.80	0.89	1.35	12.50	4.82	57.50	27.4	2.76	4.28	0.518	0.943
5	Sept. 1, 1925		PK	0.80	0.71	0.88	0.89	22.93	11.60	101.09	41.3	1.57	2.74	0.613	0.923
66	Apr. 4, 1926			0.99	1.02	0.52	0.71	17.62	8.43	66.76	32.4	2.35	4.00	0.469	0.867
88A	June 30, "			0.47	1.12	0.30	0.67	15.04	9.98	61.33	37.6	3.69	3.79	0.498	1.118
102	Aug. 15, "			0.84	0.87	0.64	0.81	19.50	12.31	84.14	43.8	2.38	3.00	0.432	1.129
188	Nov. 1, "			1.29	1.12	0.69	1.12	19.32	11.51	70.23	38.4	2.44	3.12	0.508	1.039
11	Sept. 1, 1925		N	1.00	2.22	0.79	1.78	17.16	10.44	81.82	36.5	0.85	3.16	0.794	0.794
42	Apr. 4, 1926			0.85	1.39	0.39	0.98	12.04	5.94	47.62	24.1	2.32	5.06	0.321	0.689
91A	June 30, "			0.39	1.51	0.56	0.99	6.30	6.90	38.39	30.1	4.12	4.23	0.872	0.872
95	Aug. 15, "			0.79	1.86	0.84	1.26	11.59	8.66	60.75	32.9	1.43	4.04	0.430	0.938
179	Nov. 1, "			1.45	1.93	1.01	1.60	15.57	8.39	63.55	30.6	1.43	4.08	0.467	0.906
13	Sept. 1, 1925		P	1.22	1.00	1.06	1.88	54.17	15.07	225.67	44.8	0.15	2.88	0.907	0.907
34	Apr. 4, 1926			1.04	0.86	0.77	0.80	27.97	12.76	105.89	38.5	0.63	4.35	0.507	0.828
92A	June 30, "			0.42	0.50	0.56	1.29	20.17	12.75	102.92	41.5	0.89	3.81	0.944	0.944
97	Aug. 15, "			0.99	0.80	0.81	0.87	29.30	15.60	143.96	45.6	0.39	3.57	0.659	0.860
176	Nov. 1, "			1.83	0.88	0.73	0.63	37.00	14.87	131.17	42.5	0.42	4.00	0.583	1.013

TABLE IX
RATIOS OF CARBOHYDRATE FRACTIONS TO PHOSPHORIC ACID AND POTASH, CALCULATED AS PERCENTAGES OF DRY WEIGHT OF MATERIAL

LABORATORY NO.	SAMPLING DATE		TREAT- MENT	STARCH/K ₂ O		STARCH/P ₂ O ₅		CARBOHY- DRATE/K ₂ O		CARBOHY- DRATE/P ₂ O ₅	
	WOOD	BARK		WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK
7	Sept. 1, 1925		Check	55.1	16.6	50.2	44.6	224.6	49.1	204.7	132.2
58	Apr. 4, 1926			58.2	15.7	59.7	55.0	212.0	48.8	217.7	170.9
89A	June 30, "			37.5	14.9	61.2	56.6	172.3	44.6	280.9	169.8
98	Aug. 15, "			46.3	17.2	54.4	56.1	201.6	48.4	237.2	157.8
185	Nov. 1, "			57.9	15.5	63.6	57.4	214.8	45.4	235.9	168.7
9	Sept. 1, 1925		NPK	29.7	7.5	29.7	23.6	134.2	31.4	134.2	98.3
50	Apr. 4, 1926			29.6	3.4	34.9	13.6	119.5	25.3	140.8	100.0
90A	June 30, "			11.4	4.4	16.8	23.1	93.6	20.9	137.2	108.9
94	Aug. 15, "			18.0	6.6	18.7	21.5	121.3	25.6	126.4	83.6
182	Nov. 1, "			26.0	3.6	26.4	11.7	108.7	23.8	110.5	78.1
1	Sept. 1, 1925		NP	41.5	10.9	34.3	30.3	162.2	41.7	134.4	102.1
82	Apr. 4, 1926			37.4	4.6	40.0	15.4	136.2	32.5	145.5	109.2
85A	June 30, "			15.3	6.5	20.6	23.6	119.1	31.3	160.0	112.9
110	Aug. 15, "			22.0	8.3	23.4	21.5	122.6	32.5	130.4	84.6
194	Nov. 1, "			30.0	5.8	28.5	14.4	110.0	31.7	104.5	79.1
3	Sept. 1, 1925		NK	41.6	9.8	44.8	33.1	159.6	34.2	171.6	117.5
74	Apr. 4, 1926			35.6	5.4	42.6	24.5	143.7	30.1	172.0	136.3
86A	June 30, "			16.3	5.3	31.2	30.4	99.3	24.4	190.5	139.1
106	Aug. 15, "			26.0	7.0	31.6	27.2	125.2	29.6	151.8	114.3
191	Nov. 1, "			24.1	5.1	34.5	20.6	111.0	27.4	138.6	110.5
5	Sept. 1, 1925		PK	37.4	12.6	36.3	31.8	164.8	44.8	159.9	113.1
66	Apr. 4, 1926			37.6	9.7	41.4	33.7	142.2	38.1	156.9	132.2
88A	June 30, "			30.2	8.9	55.6	35.9	123.1	33.7	226.6	135.6
102	Aug. 15, "			45.2	10.9	46.4	36.1	194.8	38.8	200.1	128.3
188	Nov. 1, "			38.0	11.1	47.1	35.9	138.3	37.0	171.2	120.2
11	Sept. 1, 1925		N	13.2	33.3	33.3	32.9	46.3	46.3	158.6	115.5
42	Apr. 4, 1926			44.5	9.8	40.8	30.1	148.3	39.7	161.5	121.9
91A	June 30, "			26.9	10.8	31.0	35.0	141.3	40.9	207.4	127.6
95	Aug. 15, "			33.3	10.9	42.3	34.3	136.1	39.7	166.8	125.1
179	Nov. 1, "			55.2	16.6	45.4	43.5	208.9	49.4	188.9	129.3
13	Sept. 1, 1925		P	55.2	15.4	48.6	55.5	208.9	46.5	184.0	167.5
34	Apr. 4, 1926			44.4	12.7	50.1	48.6	218.3	41.3	255.5	158.2
92A	June 30, "			44.4	15.9	42.1	50.6	218.3	46.3	206.8	147.5
97	Aug. 15, "			63.4	14.7	51.4	55.4	224.9	42.0	182.2	158.4
176	Nov. 1, "										

IV. Discussion of results

1. GLUCOSE AND SUCROSE

Throughout the growth cycle the concentration of glucose is very much greater in the bark than in the wood; this holds for all treatments. Both glucose and sucrose decrease rapidly during the early period of rapid growth and accumulate again as growth acceleration diminishes. But the concentration of glucose (especially in the bark) in the tissues examined of the "no-nitrogen" trees is reduced considerably below those trees which were treated with sodium nitrate.

The ratio of sucrose to glucose indicates that inhibition of invertase activity is quite marked in the trees which have not received applications of nitrogen. Table X gives these data for the bark during the period of active growth.

TABLE X
SUCROSE-GLUCOSE RATIO

TREATMENT	APRIL 4	JUNE 30	AUGUST 15
Check	1.00	1.90	1.54
NPK	0.50	0.18	0.28
NP	0.55	0.28	0.39
NK	0.52	0.30	0.55
PK	0.70	0.60	0.92
P	0.93	2.50	1.10

The addition of nitrogen has resulted in marked effect on the activity of amylase, with concomitant increase in starch utilization. The data for the bark are brought together in table XI.

TABLE XI
STARCH-GLUCOSE RATIO

TREATMENT	APRIL 4	JUNE 30	AUGUST 15
Check	12.50	29.03	23.45
NPK	1.84	3.60	3.30
NP	2.08	3.71	3.41
NK	2.63	4.28	4.36
PK	8.26	8.88	14.12
N	4.26	4.58	4.61
P	14.80	25.50	17.03

These results lend added interest to SPOEHR'S (178) view that the supply of simple sugars above that required for the normal respiratory activity is one of the factors necessary for growth, and also to LOEW'S (99)

attempt to associate blossom bud formation with a definite concentration of sugars.

2. TOTAL SUGARS

In all treatments the minimum concentration of sugars in the tissues examined is reached during the first period of active growth. Inasmuch as a minimum at this period has also been observed in other experiments (69, 122, 211), in which the external environmental factors are very different from those of the present experiment, it would appear that this phenomenon

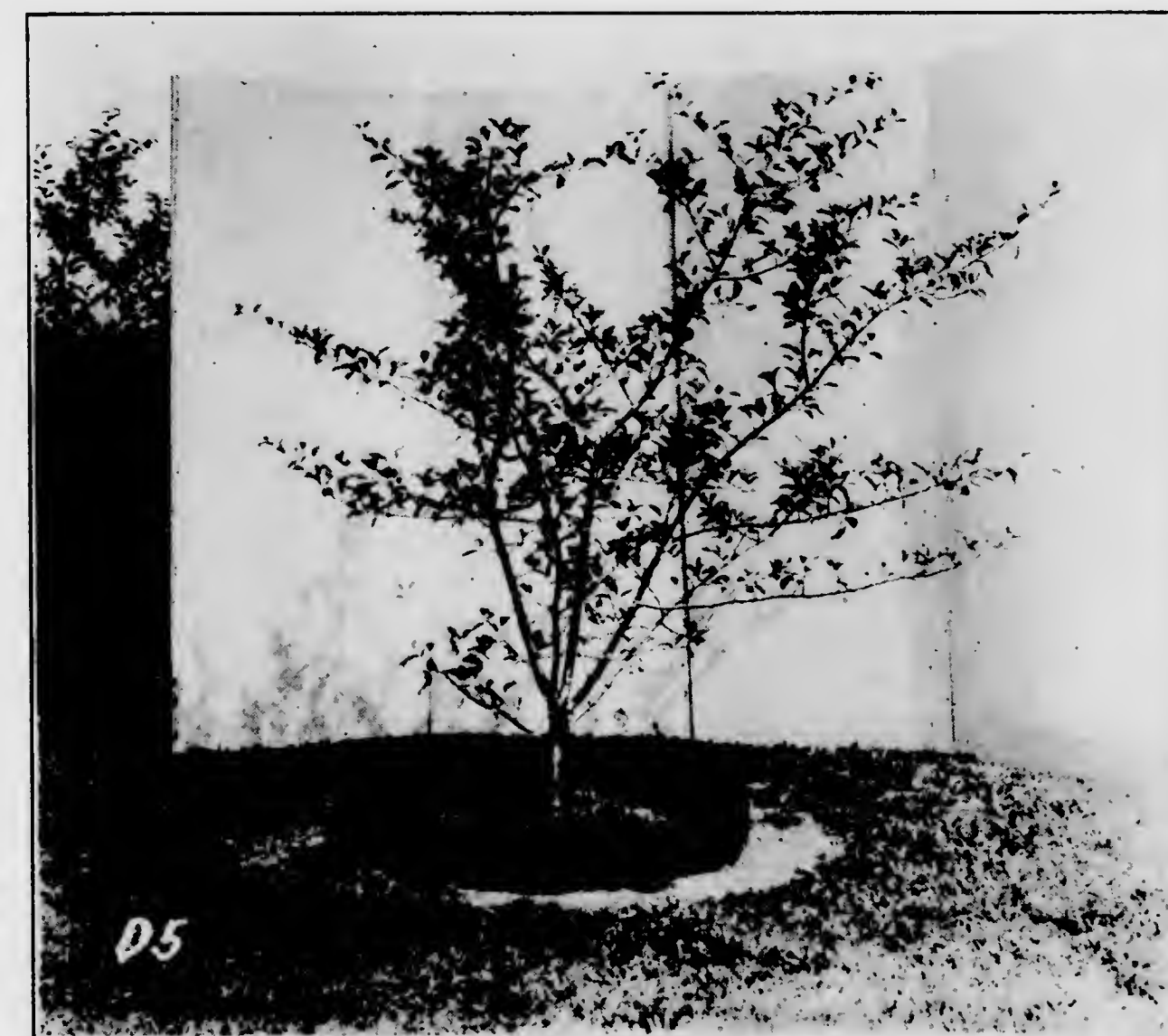


FIG. 4. Typical tree growing in sod treated with the two nutrient salts: K_2SO_4 and $CaH_4(PO_4)_2 \cdot 2H_2O$.

is independent of external factors. The concentration of total sugars in trees which did not receive added nitrogen is much lower than in the nitrogen-treated trees. The concentration of total sugars in the tissues examined as related to treatments is in the following descending order: NPK > NP > NK > N > P > check.

3. STARCH

The variations in the distribution of starch throughout the cycle follow the same general course observed in other investigations with fruit trees

(69, 122). Comparisons in this respect with other investigations have to be made with caution, however, because only a few investigators using macroscopical methods have separated bark from wood. SWARBRICK (194) concludes from a review of previous work that the seasonal change in starch content shows on the whole two maxima and two minima, the maxima occurring, respectively, at leaf fall and very early in the spring; the minima at the time of maximum leaf growth and early winter. Examination of the



FIG. 5. Typical tree growing in sod treated with the two nutrient salts: NaNO_3 and $\text{CaH}_2(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$.

results (table VI) of the present experiments shows a distinct lag of the starch changes in the wood compared with those in the bark, so that the dominant phases of the starch cycle in the wood are not the same as those for the bark. This fact has to be considered in any attempt to relate the starch cycle to other physiological phenomena, *e.g.*, to cambium activity and xylem differentiation.

The great decrease in starch which occurs in the bark—but of the nitrogen-treated trees only—is associated with swelling of the buds. This rapid utilization of starch in the spring is characteristic of the nitrogen-treated trees. Moreover, the accumulation of starch in the bark of all trees at the period of cessation of active growth and the accumulation in the wood later on in the season suggest that starch accumulation may be the cause of retardation of growth.

The starch content of the trees which received nitrogen is at a much lower level throughout the cycle than that of the trees which received no sodium nitrate applications. The trees which did not receive nitrogen show relatively small fluctuations in starch content throughout the cycle.

In this experiment there has not been observed any great deposition of starch in the cortical region of the bark found by some investigators (161). Microscopical examination at various times showed that the starch was distributed between the parenchymatous cells of the cortex, phloem, and medullary rays. The pith was packed with starch grains, although it was usually the last part to store it. A similar behavior has been noted by GARDNER (44) in the Bartlett pear. The relation of the concentration of starch to the treatments given is in the following ascending order: $\text{NPK} < \text{NP} < \text{NK} < \text{N} < \text{PK} < \text{P} < \text{check}$.

Energy relations of glucose and starch

Glucose and starch may be regarded as two closely related forms of potential energy (229). Starch is physically labile, chemically stable, non-diffusible, and non-dialyzable. Glucose, on the other hand, is chemically labile, physically stable, but diffusible and dialyzable. In the relatively low glucose concentration of the cell plasma an equally low intensity of potential energy exists, whereas starch contributes a highly concentrated form of glucose with a corresponding high intensity of potential energy. If one function of the protoplast is to act as a condensation catalyst with power of selectively removing sugars from dilute solution, this may indicate the reason why the determination of starch may be of such diagnostic value. If this view be correct, then the accumulation of starch would be the direct cause of growth retardation and not, as HARTWELL (59) suggests, the result of inhibited growth.

4. HEMICELLULOSES

The physiological function of this important class of structural cell wall substances is still obscure, but there is evidence to show that in many plants this group may function as a reserve material (93, 129, 135). Thus LEUKEL (93) found wide fluctuations in the amount of hemicelluloses in pasture and forage plants, and NIGHTINGALE (135) found a considerable decrease in

hemicelluloses in tomato plants during active growth. It must be borne in mind, however, that the sugars and starches are the more labile and, therefore, are the more indicative of immediate seasonal changes.

GERHARDT (45), NORMAN (140, 141, 142), O'DWYER (144, 145, 146), and SCHRYVER and his co-workers (28) have, in recent years, added much to our knowledge of the composition of these reserve substances. The results of these workers indicate that the hexosans (galactans, glueosans, and mannans) and pentosans (arabinans and xylans) contained in the cell walls constitute the greatest fractions in certain of the hemicelluloses. The former especially predominate in kernels of seeds poor in starch and fat. There is evidence (45, 144, 145, 146) that many of the hemicelluloses contain acid groups of the "uronic" and "glucuronic" acid type.

The hemicellulose of one-year old apple wood examined by GERHARDT (45) contained 15 per cent. lignin, 70 per cent. pentose sugars, 5 per cent. ash, and 5 per cent. moisture. No hexoses were identified. The pentosans (d-xylose and l-arabinose) were present in the molar ratio 7:1. However, TOTTINGHAM, ROBERTS, and LEPKOVSKY (210) reported that the wood of bearing spurs of apple trees contained xylose, glucose, and galactose. The preliminary hydrolysis of four products isolated from wheat bran by NORRIS and PREECE (143) consist of (1) a glucosan, and (2) substances containing pentose residues.

5. DO PRESENT METHODS OF HYDROLYSIS ADEQUATELY REPRESENT RESERVE MATERIAL?

The question has arisen as to whether our present methods of hydrolysis with weak or strong acids hydrolyze the reserve hemicelluloses of apple trees. BRADBURY and ROBERTS (13) conclude that they do not. The methods at present in use are based on the work of LECLERC DU SABLON (92) and of SCHELLENBERG (169). In apple tissues, BRADBURY and ROBERTS (13) found that these acids act only upon the young xylem, phloem, and cambium cells and the primary walls of other cells rather than upon the reserves occurring as cell wall thickenings. This may account for the results given in table VI, which show that the seasonal fluctuations in the hemicelluloses are relatively small and, therefore, do not afford a very sensitive index of carbohydrate changes throughout the cycle. This whole problem needs clarification.

In the present experiments hemicelluloses accumulate in the fall but no regularity or trend is observed when all the treatments are considered. The sequence of changes in the hemicellulose fraction parallels to some extent the fluctuations in starch content, but the changes in the concentrations of the latter afford, as already described, a much more sensitive index of the nutritive conditions. The reciprocal relation noted by MURNEEK (129) in

the reproductive organs of the apple between hemicelluloses and total sugars and also starch is not very definitely defined in the tissues examined in the present investigation. Hemicelluloses are much lower in the bark than in the wood, and are generally highest in the bark of the trees which did not receive nitrogen.

6. TOTAL AVAILABLE CARBOHYDRATES

Accumulation of total available carbohydrates occurs in all trees when growth begins to slacken. The relation of the concentration of total available carbohydrates to the treatments given is in the following ascending order: NPK < NP = NK < N < PK < P < check.

The leaves were yellowish green in the trees without nitrogen additions, a characteristic of high carbohydrate and low nitrogen conditions.

7. TOTAL NITROGEN

The distribution of the nitrogen throughout the cycle is very similar to that found in the earlier experiments of the writer (201, 202), the characteristics of which are a high concentration of nitrogen in the spring, a decrease during the period of active growth, and accumulation in the fall. The concentration of nitrogen in the trees to which sodium nitrate was applied is much higher than in the trees which did not receive any added nitrogen. Thus, in these "no-nitrogen" trees the range is from 0.151 to 0.532 per cent. in the wood and from 0.72 to 0.98 per cent. in the bark, compared with 0.44 to 0.815 per cent. and 0.89 to 0.48 per cent. in wood and bark, respectively, of the trees which received sodium nitrate. The concentrations of nitrogen in the tissues examined from the different treatments are in the following descending series: NPK > NP > NK > N > P > check.

It has already been pointed out (p. 415) that the application of sodium nitrate has resulted in a marked increase in the utilization of starch and also (but in a smaller proportion) of the "hemicellulose" fraction. The omission of nitrogen from the complete fertilizer combination (NPK) has resulted in a *decreased* and not an *increased* absorption of the remaining elements. The significance of this has been discussed by the writer in other papers (206, 208).

8. CARBOHYDRATE-NITROGEN RELATION

The relationship of this ratio to the vegetative and reproductive responses of plants has dominated the field of horticultural research during the past decade (6, 62, 63, 69, 71, 82, 83, 84, 135, 136, 155, 157, 167, 232). A summary of the American investigations on this subject up to 1924 has been given by HOOKER (71). The claims to the existence of a positive correlation between this ratio and the vegetative and reproductive responses of

plants, however, have not been allowed to pass unchallenged (6, 232). The whole problem is essentially related to the question of physiological balance.

A. PHYSIOLOGICAL BALANCE.—The concept of physiological balance in metabolic processes has played a considerable rôle in modern investigations in physiology (206). Viewing the problem with respect to the internal conditions of the plant, one may, with PEIRCE (150), take the position that from the observed operation of natural laws a shift in the equilibrium conditions within the living cell might be brought about *by any external factor* that influences the rate of any one of the multitude of chemical reactions taking place. Such disturbance in normal metabolism might be either temporary or permanent. We shall return to PEIRCE's view relative to the external influence affecting fruiting later.

B. NON-EXISTENCE OF SPECIFIC CARBOHYDRATE NITROGEN VALUES FOR A GIVEN RESPONSE.—Although KLEBS (79, 80) made many valuable observations on the effect of alteration of external factors on blossom-bud formation, it would appear that FISCHER (42) was the first to suggest the possibility of using the carbohydrate/nitrogen ratio as a means of diagnosis, although no quantitative data were reported by him. His observational data led him to conclude that flowering was associated with a very high carbohydrate/nitrogen ratio whereas a low carbohydrate/nitrogen ratio favored vegetative growth.

Although there is no suggestion in the work of KRAUS and KRAYBILL (82) of any *specific ratio* of carbohydrate/nitrogen for any response, attempts have been made to establish limiting values and even exact mathematical ratios (54) between the available carbohydrate and available (total) nitrogen for a particular response. Frequent citations of GURJAR's paper appear in the literature, but it has unfortunately been published only in abstract form. On the other hand, there is substantial experimental evidence (62, 154) to refute the association of a *specific* carbohydrate/nitrogen ratio with the flowering or the vegetative condition, even in the case of plants of the same species growing under different environmental conditions. Thus, a comparison of the carbohydrate/nitrogen ratios in the case of apple trees in New Hampshire (154) and Oregon (62) shows that the non-bearing spurs of the former and the most fruitful spurs of the latter investigations had the same carbohydrate/nitrogen ratio. Moreover, bearing spurs from the "sod" plot, which produced no fruit buds, in KRAYBILL's and POTTER's experiments (84) had the same carbohydrate/nitrogen ratio as the non-bearing spurs on the "nitrate" fertilized plot.

C. FOOD SUPPLY AND BLOSSOM-BUD FORMATION.—In the present investigation fruit-bud formation is definitely associated with such external factors

as the supply (concentration) of nitrogen, phosphorus, and potassium. This result is in accordance with empirical deductions of the earlier practical commercial growers and with the later more scientific field observations of GOURLEY (50) and of DRINKARD (36), that the supply of nutritive elements from the soil is *under most field conditions* the factor of greatest importance in fruitfulness. This fact is also shown by the results of the numerous experiments with laboratory control similar to those initiated by KRAUS and KRAYBILL.

The fact, however, that stunted plants of a grain field and fruit trees about to die from mechanical injury and disease may, in a season of insufficient rainfall, put forth bloom out of all proportion to the food supply, leads PEIRCE (150) to the view that increased absorption of mineral elements from the soil and consequently the potential supply to the *bud primordia* is not always a concomitant of fruiting. The experiments of KLEBS (79) leave no doubt that fruiting under the conditions of his experiments in vigorously vegetative plants was brought about by varying the supply of nutrients with changes in the illumination. In one of his experiments, for example, this was effected by lowering the concentration of salts and by increasing the illumination. But PEIRCE (150) would distinguish between light sufficient for photosynthesis and light as a stimulus to fruiting: "while reproduction may be accomplished without exposure to more light than sufficient for photosynthesis, the more vegetative modes will be the ones developed, the formation of the sexual organs taking place only after sufficient stimulation by light" (150). It is possible, nevertheless, that in the experiments of KLEBS and in the cases cited by PEIRCE, fruiting may have resulted when the proportions between available carbohydrates and nitrogen reached definite limiting values. More light would be thrown upon the interpretation of such phenomena if analytical data were available with which plants in the conditions just described could be compared with the results of experiments in which correlations of response with internal conditions are made.

D. EXTENSION OF CONCEPTION OF BALANCE TO OTHER NUTRIENT ELEMENTS.—The importance of the nitrogen supply in the maintenance of a proper relationship between the amount of vegetative and reproductive activities which has long been observed in agricultural practice, especially in fruit growing, can be accounted for by the fact that nitrogen is usually the primary limiting factor under field conditions (71, 167). From this viewpoint, the conception of balance may not be confined to the relations between carbohydrates and nitrogen only, but may be extended to balances between other elements or complexes, whichever may be the limiting factors to growth under the particular conditions of growth (167, 205, 206).

In the present investigation, and also in those reported by other investigators (69), carbohydrate accumulation has resulted from a deficiency of nitrogen; but accumulation of carbohydrates can result also from a deficiency of other mineral elements, especially potassium or phosphorus (37). That both potassium and phosphorus have exercised influence on the synthesis of carbohydrates in the present investigation is not open to question. The carbohydrate/ K_2O and the carbohydrate/ P_2O_5 ratios in the present investigation are shown in table IX. In relation to treatment the former follows the descending series: check = P > PK = N > NP > NK > NPK, and in the latter the order is: check > P > PK = N > NK > NP > NPK. The significance of this order of the ratios will be discussed later.

E. EXPERIMENTS OF ARTHUR, GUTHRIE, AND NEWELL.—These investigators, in an extensive investigation (6) on the carbohydrate/nitrogen ratio as related to flowering and length of day, concluded that flowering, under the conditions of their experiments, could be initiated by a long or short day, depending on the species or variety, and that the carbohydrate/nitrogen ratio for a given species varied within wide limits *independently of flowering*. From the results obtained by them, for example, on *Lycopersicon esculentum*, these investigators concluded that the "ratio of carbohydrate to nitrogen has little or nothing to do with fruiting."

In relation to these experiments by ARTHUR and his colleagues there are two important points to be considered, either of which would necessarily invalidate their conclusions just mentioned:

(1) *That in order to establish the utilization of an element or compound apart from that of mere absorption, the determination of the total amounts of materials as determined by chemical methods at any one period does not differentiate between the amount available for synthesis and the amount that may have been utilized. This distinction may be determined with any degree of certainty only by means of time-absorption curves (208).*

(2) *That conclusions with respect to the "regulation" of the absorption of an element can be made only when the effect of varying the concentration and ratios of the other principal (dominant) nutrient elements on the absorption of the element in question is known (206, 208).* In view of these facts it may be questioned whether the conclusion of these investigators that "a plant such as corn is so able to regulate the amount of nitrate taken in that it is difficult to induce this plant to take up enough nitrate to increase the total percentage of nitrogen in the plant" can be maintained.

ARTHUR, GUTHRIE, and NEWELL finally suggest "that a study of enzymes or other substances present in the very small amounts in the growing tips or elsewhere offers much more promise than gross carbohydrate and nitrogen fractions in various plant organs, in explaining the mechanism of

light in initiating flowering and fruit production in the plant." Studies of this nature, especially of the type conducted by WENT (223), are no doubt highly desirable. It is difficult to understand, however, in what way "the study of enzymes or other substances present in very small amounts in the growing tip" will assist in determining the *causal factor* of flower bud formation. There is little doubt that much of the present confusion with regard to the relationship of carbohydrates and nitrogen to flowering and fruiting is the result of a failure, in many cases, to recognize that the ratio itself is not a *causal factor* but only the resultant of numerous antecedent unknown factors.

It is difficult to escape the conclusion from the results of the present investigation that the carbohydrate/nitrogen ratio, in particular the starch/nitrogen ratio, under ordinary field conditions serves as one of the most sensitive measurable indices of physiological balance that are at present available. In the present experiment the carbohydrate/nitrogen ratio in ascending order is NPK < NP < NK < N < P < check.

F. APPLICATION OF THE LAW OF MASS ACTION.—The concept of the reversibility of the metabolic processes of the nitrogen complexes as a function of the active mass of carbohydrates accords with the known facts of the protein-sparing action of carbohydrates (180). And the work of PRIANISHNIKOW (158, 159, 160), CHIBNALL (23, 24, 25, 26, 27), and also of the writer (201, 202) suggests the possible mechanism of this reciprocal relationship between carbohydrates and nitrogen. The final and intermediate stages of the N equilibrium may be represented: "rest" $N \rightleftharpoons$ proteins \rightleftharpoons amino acids \rightleftharpoons amides \rightleftharpoons ammonia. The direction of the reactions would be to the right when the active mass of carbohydrates is low and to the left when the carbohydrate concentration is high (159).

9. ASH

The ash results present no special problem for discussion. The results consistently reflect the treatments given and are in accordance with LAEBIG's Law of the Minimum. Thus, the series in descending order of magnitude is NPK > NP > NK > PK > N > P > check.

10. POTASSIUM

A. MECHANISM OF THE ACTION OF POTASSIUM.—Recent investigations (75) point to the fact that the mechanism of the action of potassium may be very complex—the resultant of photoelectric as well as of radioactive properties. In this connection it is of interest to note that LORING and DRUCE (101) found that the potassium chloride obtained from potatoes fertilized with KCl had an atomic weight of 40.5 ± 0.1 . A qualitative test

showed this sample to be more radioactive than ordinary KCl, indicating a possible biological enrichment of K^{41} in the potato plant. This difference in the physiological effect of an isotope may be related to difference in the degree of hydration of the ions (208).

B. RÔLE OF POTASSIUM.—A review of investigations on the functions of potassium will be presented in detail by the writer in another paper. In spite of a prodigious amount of work the mechanism of the action of this element is not yet known. Why does it differ so remarkably in its action from the chemically related element sodium? Certain physiological effects of potassium, such as its stimulating (catalytic) action on the photosynthetic mechanism, are now well established (14, 48, 53, 60, 110, 115, 126, 137, 138, 170, 187, 192, 222, 227, 228). Indeed, STOKLASA and his school (187, 188, 189, 190, 191), who have carried out the most extensive and critical experiments on the subject, maintain that synthesis of carbohydrates is possible only in the presence of the potassium ion. The presence of higher concentrations of potassium in meristematic tissues than in other tissues is regarded by some investigators as evidence of a direct or indirect function in mitosis (104, 137).

C. EFFECTS OF POTASSIUM DEFICIENCY.—If the results obtained with excised leaves are comparable with the living plant as a whole, as the evidence obtained by GREGORY and RICHARDS (53) would indicate, it would appear that the effects on assimilation of a deficiency of each of the principal nutrient elements is specific for each element. Table XII summarizes the results obtained by GREGORY and RICHARDS.

TABLE XII

RELATIVE RESPIRATION AND ASSIMILATION INDICES OF BARLEY IN COMPLETE AND DEFICIENT NUTRIENT SOLUTIONS

TREATMENT	RESPIRATION	ASSIMILATION	
		LOW LIGHT INTENSITY	HIGH LIGHT INTENSITY
Complete nutrient solution	Normal	Normal	Normal
Nitrogen deficient	Subnormal	Normal	Subnormal
Phosphorus deficient	Normal	Slightly supernormal	Slightly supernormal
Potash deficient	Supernormal	Subnormal	Subnormal

Thus, only the potassium-deficient leaves showed an initial fall in the assimilation rate equally marked at low as well as high light intensity. The rate of diffusion of CO_2 into the cell up to the chloroplast surface, there-

fore, would seem to depend upon the potassium concentration, possibly through its rôle in determining the bicarbonate content of the leaf.

It is of interest to compare these results with the nutrient culture experiments of MACGILLIVRAY (106) and of JANSSEN and BARTHOLOMEW (77). The former found that a deficiency of phosphorus and the latter that a deficiency of potassium resulted in increased percentage of dry matter. From this JANSSEN and BARTHOLOMEW concluded that "up to a certain concentration the potassium ion aids carbon assimilation. If, on the other hand, the concentration of potassium is increased carbon assimilation is again decreased." These anomalous results emphasize a point that plant physiologists cannot ignore; this is that experiments planned to determine the rôle of any of the nutritive elements are of little value unless attention is paid to the question of physiological balance (208).

If, moreover, the defense mechanism of the plant against an excess absorption of an ion such as potassium is that of precipitation, as suggested by some investigators (104, 214), such regulatory ability must be very limited. The work of SEKERA (174) is important in this connection. The clear-cut evidence from SEKERA's carefully planned experiments on barley is that normal plants are able to regulate the amount of an ion absorbed in excess of that utilized in metabolism by expulsion of that element into the soil *via* the roots. This has been discussed in detail by the writer in another paper (208).

D. POTASSIUM AND TRANSLOCATION OF STARCH.—Although there is considerable evidence to indicate that potassium is effective in increasing the translocation of starch (75, 76, 77, 110, 138), experiments may also be cited (134, 176) the results of which have been interpreted as indicating that translocation of starch is independent of potassium salts. Thus, NEWTON (134) found in water cultures that almost twice the weight of potato tubers was obtained in minus-K solutions compared with those from the complete culture solutions. The weight of tops and roots, however, was slightly in favor of the latter cultures. However, in the minus-Ca series, in spite of leaving a 25-gm. seed piece attached to the seedling, the plants died. Potato tubers have relatively a very high content of potassium and a very low content of calcium. Hence, as NEWTON points out, his experimental evidence leads to the conclusion that the beneficial results of omitting potassium were due to the increased absorption of calcium. The reciprocal action of potassium and calcium has been observed in a number of experiments (208). These anomalous results accordingly may be attributed to a lack of physiological balance in the potassium cultures. The results of SMITH and BUTLER (176) may be similarly interpreted. The experiments of the latter do not indicate that translocation of starch is independent of

potassium but that the potassium present in the seed is relatively efficiently utilized during the very short duration of these experiments (21 days).

E. EFFECT OF POTASSIUM ADDITIONS IN THE PRESENT EXPERIMENTS.—

(1) *On the leaf color.*—None of the typical symptoms of potassium deficiency, viz., yellowish green leaves (the color is usually dark green if sodium is also deficient) with purple veins or browning of the periphery, were observed in any of the trees.

(2) *On the enzyme system.*—Many experiments (33, 40, 41, 175, 184, 193) indicate that potassium has a marked effect on the enzyme systems of the plant and that the effects are specific for different enzymes. In the present experiments potassium additions have increased the activity of amylase and of invertase (table VI). The marked effect of potassium in increasing the utilization of starch in these experiments is also apparent from the data given. Thus, the starch/ K_2O ratios are in the following ascending order with respect to treatments: NPK < NP < NK < N < PK < P < check. It is of interest to note that REED and HAAS (163) observed that orange trees can function with less potassium than they ordinarily absorb. Trees receiving no added potassium were able to grow for 17 months subsisting presumably on the potassium in the trees at the time they were planted.

(3) *On the absorption of nitrogen and phosphorus.*—Potassium additions have increased the absorption of both nitrogen and phosphorus in the tissues examined (205, 206) and, as a later paper will show, this statement may be generalized to the absorption of these elements by the entire tree.

(4) *On the potassium/nitrogen ratio.*—WALLACE (219, 220) has emphasized the significance of this ratio. In his sand cultures, leaf scorch (tip burn) of trees growing with insufficient potassium increased with each increment of nitrogen in the nutrient solution; at relatively high K/N ratios leaf scorch (tip burn) did not develop. Bronzing of the leaves of citrus trees growing in nutrient solutions having a low K/N ratio was also reported by REED and HAAS (163).

Certain investigators (39, 77) have noted a reciprocal relationship between the content of the plant with respect to nitrogen and potassium. The whole question of the mechanism of the reciprocal relationship between nitrogen, phosphorus, and potassium in relation to their absorption by plants has been discussed in detail by the writer elsewhere (206, 208). It may be pointed out here, however, that no generalizations are possible with respect to the K/N ratio because the absorption of these elements by the plant is a function of the level of nutrition, i.e., rate of supply of nutrients (206, 208). Accordingly, results may show either a positive (as in the present experiment) or a negative correlation (39, 77) with respect to the amount of these elements absorbed by a plant.

11. PHOSPHORUS

A. *RÔLE OF PHOSPHORUS IN RESPIRATION.*—The experiments of both LYON (102) and SPOEHR (179, 181) *in vitro* indicate that phosphorus may act as an accessory accelerator of respiration. The starch/ P_2O_5 and the carbohydrate/ P_2O_5 ratios (table IX) of the trees of the various treatments in the present field experiments also suggest such an effect. The isolation of a reducing hexosemonophosphate by NEUBERG (131, 132) from bottom yeast and by BARRENSCHEEN and PANY from *Elodea canadensis* (12) affords very positive evidence that phosphates play a rôle in the intermediate stages of the carbohydrate metabolism of plants.

B. *ABSORPTION OF PHOSPHORUS.*—In the present experiments the absorption of phosphorus by trees which did not receive phosphorus application and by those trees which received phosphorus additions unaccompanied by the other principal nutrient elements is relatively low. A significant increase in the absorption of added phosphorus has occurred only by the addition of potassium or nitrogen or both, i.e., in the NP and NPK treatments. Since a rapid "fixation" of PO_4^{3-} ions takes place in this Hagerstown clay loam soil (197), this increase in the absorption of phosphorus may be interpreted as the result of an increase in the solubility of phosphorus following a decrease in the already existing potential difference between the micellar and intermicellar soil solution by the K^+ and NO_3^- ions, in accordance with the GIBBS-DONNAN law (1, 49, 116, 183, 185, 208).

C. *STARCH/ P_2O_5 RATIO.*—Both the starch/ P_2O_5 and the available carbohydrate/ P_2O_5 ratios indicate that phosphorus may act as an accessory accelerator of respiration only if adequate supplies of nitrogen and potassium are present. Hence, it follows that in soils, such as the Hagerstown clay loam used in this experiment, which are relatively low in nitrogen and phosphorus albeit high in available potassium, the application of a carrier of phosphorus (P) or of phosphorus and potassium (PK) would be ineffective to permit sufficient utilization of carbohydrates for normal growth and reproduction unless nitrogen is also applied.

D. *N/ P_2O_5 RATIO.*—In the leaves of *Vitis vinifera*, LAGATU and MAUME (87) found a linear relationship between the nitrogen and phosphorus contents. This would be expected if all the phosphorus is in organic combination as nucleic acid, lecithin, and allied substances. The nitrogen and phosphorus would then be present in the same molecule. This linear relationship does not exist in the tissues examined in the present experiment, for wide seasonal changes in the N/ P_2O_5 ratio occur in all treatments. This indicates that the large and variable proportions of the phosphorus are present as nitrogen-free compounds.

12. WATER RELATIONS: PHYSIOLOGICAL DRYNESS OF TISSUES

The position of the water balance within a plant is determined by the power of the roots to maintain the supply and the power of the leaves to resist excessive loss. The rate of water loss, if the plants do not differ internally in some manner affecting transpiration, will depend upon the nature of the leaves and the area of foliage produced. Many experiments (22, 58, 98, 173) indicate that the nearer to the optimum the physiological balance of the nutrient solution the smaller becomes the transpiration per unit of dry matter produced.

A. POTASSIUM AND PHYSIOLOGICAL DRYNESS OF TISSUES.—From the observation that potassium-starved fruit trees contained less water than plants receiving potassium additions, WALLACE (221) postulates that physiological dryness of tissues may be a characteristic of plants deficient in potassium. JAMES (76) also found a significant correlation ($P < 0.01$) between potassium and water content throughout the growth period of *Solanum tuberosum* in the presence of chloride ions but not in their absence, and GREGORY and RICHARDS (53) found even a decreased water content associated with additional potassium at moderate concentrations. These facts would seem to indicate that other causes than osmotic pressure and solution tension are at work. Nevertheless, the influence of such a strongly hydrated element as potassium upon the swelling properties of gels must be considerable. These results are of interest with respect to the relation of potassium to chlorophyll formation and carbohydrate synthesis discussed earlier in this paper. In this connection, it is interesting to note that the work of DASTUR and BUHARIWALLA (32) shows a positive correlation between the chlorophyll content and water content. Both rise as the leaf grows to maturity and both fall (the water content most) as the mature leaf grows old.

In the present experiments, nitrogen appears to be the greatest factor in decreasing physiological dryness of tissues (table V). This may be due to the influence of sodium nitrate additions on the increased uptake of potassium. The leaves and one-year and season's branch growth of trees receiving additions of all three nutrients (NPK) have the highest water content. The series in descending order is: NPK > NP > NK = N > P > check.

B. BAKHUYZEN'S PHYSICO-CHEMICAL INTERPRETATION.—BAKHUYZEN (10) has sought to interpret the facts of permeability, of ecological behavior, of differentiation and adaptation, and also of the life cycle of plants from the colloid chemical view-point. The water content, therefore, plays an intrinsic rôle in this theory. If the data of MOMMSEN (124) and NAYLOR (130) can be generalized, "external" protoplasm has an isoelectric point

at a pH of 4.2–4.5, whereas that of the nucleus is much higher, viz., pH 5.0–5.5. There exists, therefore, a relatively high ζ -potential between "external" protoplasm and the nucleus; in other words, the peripheral plasma is more negatively charged than the perinuclear part. Accordingly, BAKHUYZEN postulates that, as the growth of the cell proceeds, the hydration capacity of the "external" protoplasm increases (increase of negative charge), whereas that of the "internal" protoplasm or perinuclear plasma decreases (decreases of negative charge or increase of positive charge). Since the minimum hydration of a colloid is at its isoelectric point, a coagulation of the internal plasma with consequent loss of hydration water and absorbed solutes occurs as growth proceeds. In this way the vacuole is formed. As the cell grows the difference between the charge (ζ -potential) of the "external" and that of the "internal" protoplasm increases up to the point when the cell begins to age. If the negative charge on the outside of the protoplasm is destroyed, solutions of acids, bases, or salts will nevertheless tend to stabilize the colloid. BAKHUYZEN argues that since OSTERHOUT (147) finds that death of protoplasm results in loss of isoelectric charge, there must, in long-living cells, be a force which keeps up this charge during the life of the cell. He postulates that the source of this counteracting force is probably the growth hormones, which increase the hydration capacity of the protoplasm during growth and tend to keep it up after growth in volume is over (56, 223). The withdrawal of water and salts from the vegetative parts (9) is thus accounted for.

V. Summary

Standard apple stocks vegetatively propagated from a single parent and whip-grafted to scions from a tree of known history were planted in a virgin soil contained in 5 foot by 5.5 foot boiler plate cylinders. These cylinders, 42 in number, were sunk in the ground but were isolated from the surrounding soil by means of a 6-inch layer of crushed limestone. Two years after planting, the trees were subjected to differential fertilizer treatments by the application of different combinations of the pure salts NaNO_3 , $\text{CaH}_4(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$, and K_2SO_4 , applied in very small (low) concentrations in the spring of each year.

Marked differences in response to treatments were visible within a few months and became progressively intensified as the experiment continued. During the years 1925–1926, analyses of the one year and season's branch growth with respect to certain elements or groups of elements were made at critical periods in the seasonal growth of the trees under sod. The bark and wood were separately examined. The reproductive responses are indicated by the number of blossom clusters formed during this period. Under cultivation: NPK, 353; NP, 334; N, 71; PK, 28; NK, 17; P, 13; check, 6.

Under sod: NPK, 328; NP, 145; NK, 102; N, 21; PK, 0; P, 0; check, 0. Branch elongation followed the same order relative to treatment.

The analytical results on the one year and season's branch growth show that:

1. Both "glucose" and sucrose decrease rapidly during the period of active growth and accumulate again when growth acceleration diminishes; this accumulation of simple sugars appears to be a necessary precursor of growth.
2. The concentration of simple sugars in the case of trees which did not receive any sodium nitrate applications is much lower than in the nitrogen-treated trees.
3. Inhibition of invertase activity is marked in the case of the trees which did not receive nitrogen.
4. The starch cycle in the wood lags behind that of the bark. Bud swelling, especially in the nitrogen-treated trees, is associated with a great decrease of nitrogen in the bark.
5. Storage of starch up to the fall in the no-nitrogen trees is relatively very great, but the accumulated starch reserves of the trees which received applications of sodium nitrate rapidly diminish as growth proceeds. In relation to the different treatments, starch follows the ascending order $\text{NPK} < \text{NK} < \text{N} < \text{PK} < \text{P} < \text{check}$. Storage of starch is intimately connected with cessation of active growth and appears to be the cause of growth retardation in these experiments.
6. The energy relations of glucose and starch are examined and discussed.
7. The literature on the physiological function of the hemicelluloses as reserve material is briefly reviewed. In the present experiments the seasonal fluctuations of this group are relatively small.
8. As in the case of starch, the total available carbohydrates in relation to the different treatments follow the same ascending series: $\text{NPK} < \text{NP} < \text{NK} < \text{N} < \text{PK} < \text{P} < \text{check}$.
9. Total nitrogen decreases rapidly during the period of active growth and accumulates in the fall. The concentration of nitrogen in relation to the treatments follows the descending series: $\text{NPK} > \text{NP} > \text{NK} > \text{N} > \text{P} > \text{check}$.
10. The addition of nitrogen as sodium nitrate results in a great increase in the utilization of starch. Nitrogen concentration is the principal factor that determined, in these experiments, the utilization of accumulated starch; but increased phosphorus and potassium concentrations in the tissues examined also stimulate additional utilization resulting in increased fruit-bud formation.

11. The literature of the carbohydrate/nitrogen ratio (and also of the starch/nitrogen ratio) in relation to the vegetative and reproductive responses of plants is examined and discussed. It is pointed out that, although no mathematical ratio in the sense of the existence of a specific carbohydrate/nitrogen ratio for a particular response exists for plants growing under different meteorological conditions, nevertheless, as the present experiment indicates, this ratio serves, under the conditions ordinarily met with in the field, as one of the most sensitive measurable indices of physiological balance that are at present available. In the present experiment this ratio shows a definite gradient relative to the treatments in the following ascending series: $\text{NPK} < \text{NP} < \text{NK} < \text{PK} < \text{P} < \text{check}$.

12. The percentage of ash in relation to treatments is in descending order: $\text{NPK} > \text{NP} > \text{NK} > \text{PK} > \text{N} > \text{P} > \text{check}$.

13. The literature with respect to the rôle of potassium in plant nutrition is briefly discussed. It would appear that the mechanism of the action of potassium is very complex and is probably the resultant of photoelectric as well as radioactive properties.

14. Although the soil used in these experiments has a high potassium content (3.96 per cent. K_2O), the application of K_2SO_4 increased the potassium content of the tissues which has resulted in increased utilization of starch.

15. The reciprocal relationship between potassium and nitrogen of plants found by some investigators is discussed. A positive and not a negative correlation between these elements is found in the present experiment.

16. The application of phosphorus as $\text{CaH}_4(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$ also resulted in increased utilization of starch. Phosphorus appeared to have assisted in the oxidation of the simple sugars.

17. The amount of phosphorus absorbed by trees which received no applications of a phosphorus carrier, and also by those trees which received phosphorus additions unaccompanied by one of the other nutrient elements, is relatively small. The reasons for this are discussed. Applications of potassium and also of nitrogen have increased the absorption of phosphorus.

18. Both the starch/ P_2O_5 and the available carbohydrate/ P_2O_5 ratios indicate that phosphorus may act as an accessory accelerator of respiration only if adequate supplies of nitrogen and potassium are present.

19. Although some investigators find that physiological dryness of tissue may be characteristic of potassium-deficient plants, in the present experiment nitrogen has been the greatest factor in decreasing physiological dryness of tissue. Thus, the amount of water in the tissues examined follows the descending series: $\text{NPK} > \text{NP} = \text{N} > \text{NK} > \text{PK} > \text{P} > \text{check}$.

20. The literature on many phases of the interrelationships between the plant and the soil pertaining to the present study is reviewed and examined.

The budding, grafting, and general care of the trees were in charge of Dr. R. D. ANTHONY and Mr. J. H. WARING, of the Department of Horticulture. The growth measurements were made by Mr. LEIF VERNER and Mr. R. H. SUDDS, also of the Department of Horticulture.

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SOME RECENT ADVANCES IN BIOLOGICAL CHEMISTRY*

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A review of the biochemical literature for the past year or two shows that the biochemist has made remarkable progress in many fields of work. Advancement has been so rapid in certain fields that it would appear that our textbooks and teaching may have to be completely revised. I have been impressed with the remarkable advancement that has been made in the past few months with reference to the separation, isolation, and determination of structure of many biological compounds.

I have made no attempt to make a survey of all biochemical research for the past few months but I shall endeavor to describe a few outstanding researches in three or four fields of activity which have appealed to me as being of interest and importance to biologists and workers in medical fields, as well as to biochemists.

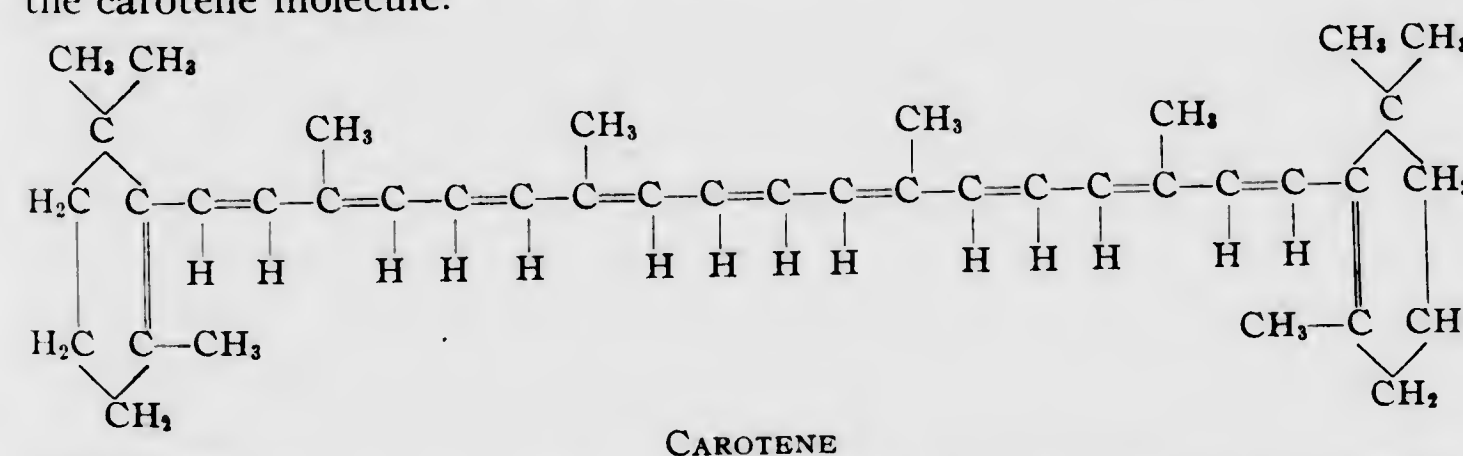
To one who has worked in the vitamin field for a number of years, the developments in the past few months have been almost spectacular. I shall describe briefly a few developments in this interesting field of research.

Vitamin A

Karrer and co-workers in Zürich have made an exhaustive series of studies relative to the chemical relationship existing between the pigment carotene and vitamin A (1). Carotene is known to exist in two isomeric forms, viz., α -carotene and β -carotene. The former is optically active and shows an absorption band at $511m\mu$ while the β variety is optically inactive and shows an absorption band at $521m\mu$ (2).

It is quite apparent that the carotene molecule ($C_{40}H_{56}$) is split, in the liver, to vitamin A, which has an empirical formula of $C_{20}H_{30}O$. Drummond and co-workers (3) announce that their work confirms the work of Karrer.

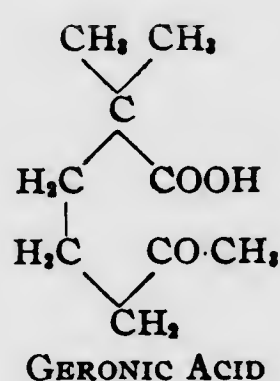
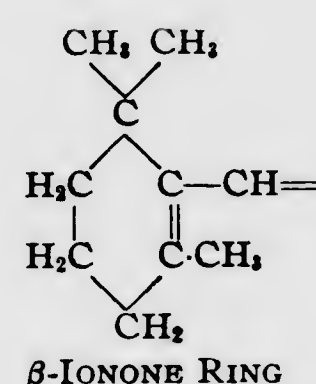
Beta-carotene yielded geronic acid when treated with ozone. Since β -ionone gives about the same yield of geronic acid by the same (ozone) methods, it is concluded that the following symmetrical formula represents the carotene molecule.



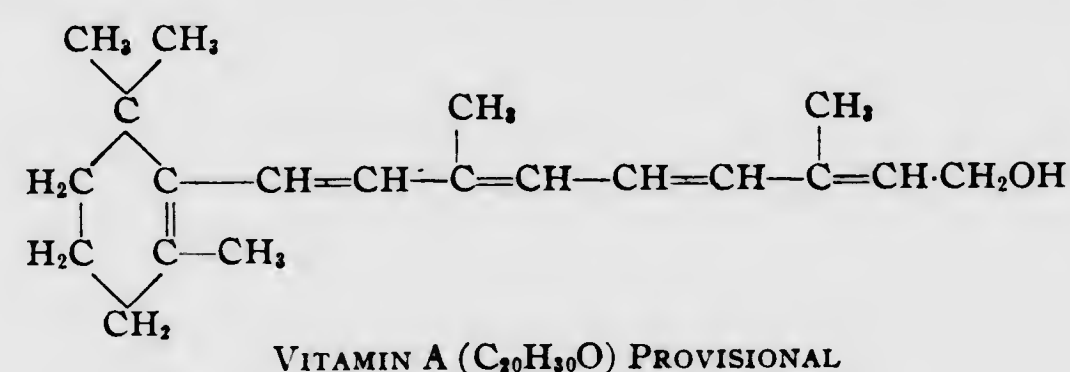
* Delivered at the joint meeting of the Divisions of Biological and Medicinal Chemistry and of Chemical Education at New Orleans, March 30, 1932.

Olcott and McCann (4) believe they have proof that carotene is changed into vitamin A in the liver by means of an enzyme which they call "carotenase."

Karrer and associates examined fish livers and found that they could isolate a substance which was more potent than carotene. The compound contained C, H, and O and yielded geronic acid. The molecular weight was about 320 and it was concluded that the vitamin A molecule contains but one β -ionone ring.



The work of Karrer and Drummond and co-workers indicates that the vitamin A molecule is about one-half the size of the carotene molecule and that vitamin A is an unsaturated primary alcohol containing the β -ionone ring. While the formula is not established definitely at the time this is being written, it would appear that the following formula is probably correct with the possible exception of the positions of the unsaturated linkages.



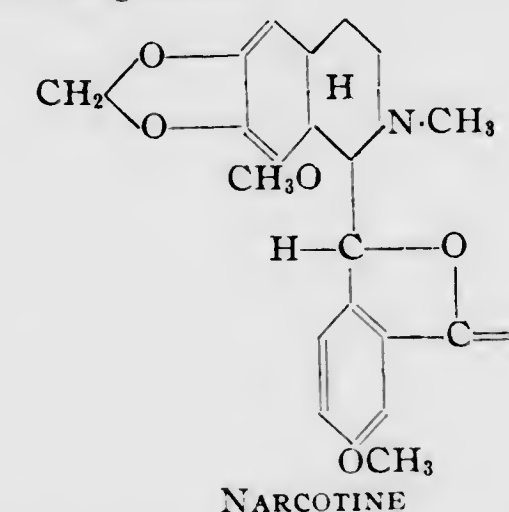
Vitamin B

Considerable work has been done to show that the vitamin B complex may consist of five or more fractions. The existence of the antineuritic fraction, B_1 , has been established definitely and vitamin B_2 is recognized as a definite entity. These vitamins are also known, in this country, as B and G, respectively. Eddy and co-workers contend that a third factor (B_3) is necessary for growth in pigeons, while Miss Reader of London believes that there is a fourth fraction of the old B complex which is necessary for normal growth in the rat. Miss Chick, also of London, is of the opinion that she has obtained evidence that a fifth fraction exists which is necessary for rat growth and which differs from B_4 in stability.

Windaus and co-workers (5) have just announced the isolation of the antineuritic fraction (B or B_1) in crystalline form. Using modifications of the methods used by Jansen and Donath, Seidell and Peters, two types of crystalline products were isolated, one as the hydrochloride and one as the picrolonate. He ascribes the tentative formula, $C_{12}H_{17}N_3OS$ to this highly potent crystalline product. This compound was active in daily doses of from 2 to 4 gamma (2/1000 to 4/1000 of a milligram). The most potent preparations reported previously were those of Jansen and Donath, which were active in doses of from 7 to 9 gamma daily.

Vitamin C

It would appear that we are soon to be able to ascribe a chemical formula to vitamin C (the antiscorbutic vitamin) if the conclusions of Rygh, Rygh, and Laland (6) are correct. These workers, publishing from the University of Upsala, describe the isolation of the alkaloid narcotine from oranges, cabbages, tomatoes, and potatoes. In unripe oranges narcotine is present in fairly high concentration but it appears to diminish as vitamin C increases during the ripening process. Narcotine, which of itself is inactive as an antiscorbutic agent, is capable of alleviating scurvy symptoms after treatment with ultra-violet light. When the ortho methoxy groups of narcotine were split off, the ortho diphenol derivative was formed. This compound proved to be efficient in preventing typical scorbutic symptoms, in doses as small as 20 to 30 gamma.



The continuation of this work will be watched with great interest but it must be corroborated by other laboratories before being accepted. There are certain phases of the feeding and pathological technic that appear to be open to question. For the present, therefore, we can only state that it may be possible that narcotine is the parent substance of vitamin C in much the same way that inactivated ergosterol is the parent substance of vitamin D.*

* W. A. WAUGH and C. G. KING of the University of Pittsburgh have recently announced that they have been successful in isolating vitamin C in pure form. They question the accuracy of the work of RYGH, RYGH, and LALAND and contend that vitamin C is similar to or identical with hexuronic acid.

Vitamin D

The most outstanding development with reference to vitamin D has been the preparation of this factor in crystalline form.

Askew and co-workers in England (7), Windaus in Germany (8), and Reerink and Van Wijk in Holland (9) have described the preparation of crystalline vitamin D of high potency. The active product is isomeric with ergosterol, contains three sets of double bonds and a hydroxyl group. The English workers have applied the term "calciferol" to their crystalline product. These workers make the ester of the activated sterol by adding 3,5-dinitrobenzoyl chloride in dry pyridine. The ester is finally crystallized out of acetone, is lemon yellow in color and melts at 147–149°. After hydrolysis of the ester, the vitamin is crystallized out of methyl alcohol in colorless crystals which melt at 114–117°. The D₂ of Linsert and Windaus seems to be identical with calciferol. Heating caused calciferol (D₂) to change to an inactive product which no longer possessed physiological activity. All these active preparations were extremely potent.

These researches seem to have established the fact that, while a number of changes are produced when ergosterol preparations are activated with ultra-violet light, there appears to be but one antirachitic substance formed, which is called D₂ by Windaus and calciferol by Askew and co-workers.

Physiological Oxidations

In 1926 Philip and Grace Eggleston of London (10) described a labile organic phosphorus compound which they isolated from muscle tissue and to which they gave the name "phosphagen." In 1927 Fisk and Subbarow (11) described experiments which indicated that phosphagen is a compound of creatine and phosphoric acid and that phosphagen breakdown affects the buffering power of muscle tissue.

It was observed that phosphagen disappeared during contraction but reappeared in the recovery period following stimulation. It was noted that lactic acid formation seemed to follow, rather than accompany, stimulation.

In the meantime Lohman (12) had found another muscle substance which was identified as a compound of adenylic and pyro-phosphoric acids, which has been identified recently as a part of the co-enzyme system in carbohydrate hydrolysis.

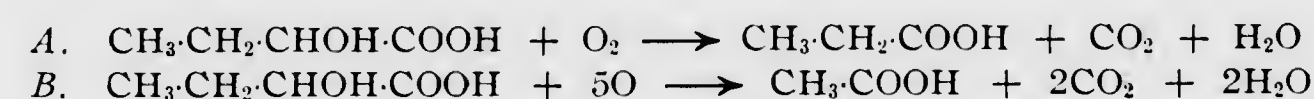
It remained for Lundsgaard (13) of Copenhagen to discover (1930) that muscles could be poisoned with iodoacetic acid, resulting in complete inhibition of lactic acid formation but without loss of contractility. Vertebrate muscles when thus poisoned may contract for a time, accompanied by phosphagen breakdown but without the formation of lactic acid. Such muscles lose their power of recovery and cease to become excitable. Under these conditions phosphagen breakdown is complete with no evidence of attempts at re-synthesis of phosphagen.

In normal, unpoisoned vertebrate muscle, the muscle does not lose its excitability and lactic acid formation is noted during the recovery period, accompanied by the re-synthesis of phosphagen. The same general observations have been made with crustacean muscle except that the phosphagen molecule consists of arginine and phosphoric acid rather than creatine and phosphoric acid.

The recent statements of Hill and Parkinson (14), Lundsgaard and others have revolutionized the older viewpoints relative to the mechanism of muscle contraction. Present data indicate that most, if not all, of the energy of contraction comes from the breakdown of the phospho-creatine complex (phosphagen). The energy of lactic acid formation appears to be utilized quite largely in the re-synthesis of phosphagen. This is an endothermic process. The formation of lactic acid, although exothermic in character, was lost sight of for some time, owing to the utilization of this energy in phosphagen synthesis. It would appear that rigor is not due, primarily, to accumulation of lactic acid, since rigor often precedes lactic acid formation. Hill (15) and Himwich (16) have made the most recent reviews of the literature in this field.

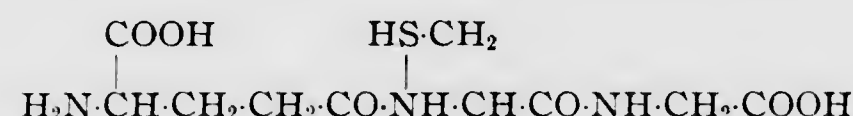
If Witzemann of Wisconsin is correct we may be forced to revise our conceptions of certain phases of biological oxidations. This author (17) has studied the mechanism of oxidation of fatty acids with particular reference to α -hydroxy acids. He finds that the rule of Popoff, *viz.*, that α -hydroxy acids undergo oxidation by loss of one carbon atom, is not true for α -hydroxybutyric acid.

Two types of breakdown are possible:



Working with the higher α -hydroxy acids he finds that these may be induced to lose two carbons at a time also. He obtained "shifts" in points of rupture by varying the chemical environment. His results indicate that the loss of one carbon is due to oxidation of the α -keto acid, while the loss of two carbons follows the oxidation of the enol isomer. He suggests that other "shifts," with reference to points of rupture, are possible and feels that α oxidation, in the animal organism, is not necessarily accompanied by loss of but one carbon atom.

Hopkins and co-workers (18) have continued their work in tissue respiration in which they submit additional evidence that the transportation of hydrogen to molecular oxygen by the "glutathione path" may represent a significant and specialized aspect of tissue oxidation. Nicolet (19) has published evidence to show that glutathione is γ -glutamylcysteyl-glycine, rather than γ -glutamylglycyl cysteine.



Mattill's work on anti-oxidants and auto-oxidation of fats (20) has raised a number of interesting scientific and industrial questions, which can only be answered by continued research. Continuing his studies of auto-oxidation as influenced by the presence of anti-oxidants and pro-oxidants (promoters), Mattill has worked with lard-cod-liver oil mixtures and has measured the induction periods, using a modification of the Greenbank-Holm apparatus. Phenol, metacresol (and other cresols) were inactive as anti-oxidants. The dihydroxy benzenes of the ortho and para types possessed marked anti-oxygenic properties but the meta type was inactive. Of the trihydroxy benzenes, pyrogallol was very active as an anti-oxidant, while phloroglucin was but feebly active.

Hydroquinone, which is very active, was inactivated by forming the diacetate, indicating that the hydroxyl groups are responsible for the anti-oxidant effect. *Alpha*-naphthol was ten times more active than *beta*-naphthol and *alpha*-naphthoquinone was more active than its *beta* counterpart.

Mattill suggests that biological oxidations may be governed, in part, by the presence of traces of oxidation inhibitors. He points to the fact that epinephrine is an ortho dihydroxy compound and thyroxine a para compound. Carotene is more stable (when fed as a source of vitamin A) in peanut oil than in mineral oil. He explains this by postulating the presence of anti-oxidants in the peanut oil. He appears to ignore the fact, however, that the mineral oil may not have been absorbed, resulting in a loss of carotene due to excretion of unabsorbed carotene.

Enzymes

Since Sumner's announcement (21) in 1926 that he had succeeded in preparing a very active crystalline urease, considerable work has been published to indicate that urease and other enzymes are capable of existing in crystalline form and that the crystalline structure consists of globulin or a type of protein closely resembling globulin. Northrup and Kunitz (22) have described a crystalline pepsin and, during the past year, have announced (23) the preparation of a crystalline trypsin. A similar announcement regarding the preparation of a crystalline amylase was made during the past year by Caldwell, Booher, and Sherman (24) of Columbia University.

Waldschmidt-Leitz and Steigerwaldt (25), however, contend that the crystalline protein is not of itself enzymic, for the reason that they were able to subject crystalline urease to proteolytic hydrolysis until no protein precipitation was possible by sulfosalicylic acid, without destroying ureo-

lytic activity. Since hydrolysis did not inactivate the enzyme, these authors are inclined to the belief that the crystalline protein is but a carrier for the enzyme and that the protein is probably no more than an accessory concomitant of the pure (urease) enzyme.

In direct opposition to this view are those of Zakowski (26), who describes similar experiments in which the activity of crystalline urease diminished during hydrolysis of the crystallized protein. He concludes that his data point to the identity of crystalline globulin with urease.

Within the past few weeks Waldschmidt-Leitz and Reichel (27) describe experiments which tend to show that pure active amylase preparations are protein-free.

No review of enzyme research can be made without referring to the recent investigations of Dubos and Avery (28). These writers describe a well-planned, systematic series of experiments on Type III Pneumococcus. It has been known that the capsule of this organism consists of a polysaccharide. Attempts to hydrolyze the polysaccharide with HCl were accompanied by loss of reactivity in specific antisera. These results supported their conviction that the capsular polysaccharide was responsible for the type specificity of Type III Pneumococcus.

To obtain more evidence they turned to the enzymes and for several years a systematic search yielded no results. Finally an organism was obtained from a New Jersey cranberry bog which possessed the power of hydrolyzing the capsular polysaccharide.

After a very interesting series of experiments they were able to isolate a relatively potent endocellular enzyme which was specific in removing the capsular polysaccharide in the presence of serum and at a pH close to 7.

As a fitting climax to this excellent research, they injected potent preparations of this enzyme into mice and found no harmful effects. Subsequent studies showed that the new enzyme preparation protected mice against infection with Type III Pneumococcus. In mice in which infection was established, the enzymes exerted a favorable influence.

Dubos and Avery feel that the reaction is a chemical rather than an immunological one. Their data indicate that the capsule is a decisive factor in determining the fate of pneumococci in the animal body and that it is vulnerable to specific agents other than anti-bodies. While they have no direct proof for their theory, they feel that the disappearance of the polysaccharide capsule may prepare the organism for phagocytosis.

While the authors, in their conservatism and modesty, say little or nothing regarding the possibility of preventing and curing pneumonia in humans—it is my humble opinion that the medical profession is one step nearer the control of another dread disease as a result of assistance and coöperation of the handmaidens of medicine—bacteriology and biochemistry.

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THE EFFECT OF "LOW VOLTAGE" X-RAYS ON THE ELECTROPHORETIC MIGRATION VELOCITY, VIABILITY AND pH OF ESCHERICHIA COLI SUSPENSIONS^{1,2}

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Bacteria under normal environmental conditions have a negative electrophoretic potential. Recently two of us, Lisse and Tittsler (1931), reported that the irradiation of *Escherichia coli* with ultraviolet or even Mazda bulb rays affected the electrophoretic migration velocity, viability, lysis and pH of an aqueous suspension. As a result of these studies we came to the conclusion that when these rays kill bacteria they first stimulate, a process accompanied by an increase in the electrophoretic migration velocity, and later destroy, a process accompanied by a decrease in this velocity. In brief, this work suggested the hypothesis that stimulation and injury are reversible processes in which the electrophoretic potential tends to return to normal, whereas death is an irreversible process in which there is no such return. The validity of this hypothesis may be questioned in view of the fact that Winslow, *et al.* (1923) reported "that heat-killed bacterial cells exhibit essentially the same curve of migration velocity as that of living cells."

According to the principles of photochemistry one is led to believe that certain effects, for example, those observed after

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irradiation with ultraviolet rays, are produced by a very specific range of wave lengths, and that rays of either longer or shorter wave lengths are less active. In this connection, however, it should be noted that Bovie (1916) showed that the Schumann rays are more reactive than the longer ultraviolet rays.

Warren (1928), in a review of "The Physiological Effects of Röntgen Radiations upon Normal Body Tissue" called attention to the injurious effects of these radiations upon body tissues, cell structures and life processes, and also noted that "it has been pointed out by many authors that these rays must be absorbed in order to be effective." Thus, it would seem that the injurious effects upon bacteria which have been noted by many workers must have been caused by absorbed radiations. This made us believe that if x-rays are absorbed by bacteria they may produce changes similar to those produced by ultraviolet irradiation.

The purpose of this investigation was to test the hypothesis advanced above by a direct determination of the effects of "low voltage" x-rays upon the electrophoretic migration velocity, viability, and pH of an aqueous suspension of *Esch. coli*.

So far as we are aware the literature contains no direct reference to the effect of x-rays upon the electrophoretic potential of bacteria. However, Fiorini and Zironi (1914) stated that the agglutinability of typhoid bacilli was not modified by x-rays. This would suggest no change in the electrophoretic potential according to the reports of two of us, Tittler and Lisse (1928) and Lisse and Tittler (1931).

The effects of x-rays upon the viability and physiological behavior of bacteria, and other microorganisms, have been studied extensively since Minck (1896) reported that typhoid bacilli were not injured by low voltage x-rays. The experimental methods and results of the early investigations have been reviewed thoroughly and adequately by Russ (1906), Klövekorn (1925) and Trillat (1927). The results of these studies differed greatly. Although some observers reported retarded growth, suppression of certain physiological characteristics such as pigment production, and even marked lethal action, there were many others who found that x-rays had little, if any, effect upon bacteria.

It would appear that x-rays either interfere with the growth and normal development of bacteria and yeasts, or produce a decided lethal effect according to many of the recent reports among which are those by Lacassagne (1928), Holweck (1929), Clark and Boruff (1929), Pauli and Sulger (1929), Holweck and Lacassagne (1930), Ellinger and Gruhn (1930), Wyckoff (1930a and 1930b) and others. However, Beckwith, *et al.* (1930) reported that the bacteria were not killed in their experiments.

Curie (1929) and Glocker (1931) have discussed the bactericidal action of x-rays from the quantum standpoint.

TECHNIQUE

Preparation of the suspension. The strain of *Esch. coli* used in the previous study of the effects of ultraviolet irradiation and which gave practically constant electrophoretic migration velocities during two years was used in this investigation. The cultures were grown on proteose-peptone agar slants of approximately pH 6.8 for twenty-four hours at 37°C. The twenty-four hour period of incubation was chosen because Shibley (1924) showed that the quantity of charge varied until the culture was about eighteen hours old, after which it remained rather constant. The growth was removed with a small volume of distilled water by a gentle rotary movement of the culture tube. This suspension was filtered through cotton, centrifuged at about 3,000 r.p.m. for one hour and the cells resuspended in distilled water. The washing was repeated twice more as advocated by Northrop and DeKruif (1922). A very concentrated suspension was prepared for irradiation as the structure of the x-ray apparatus did not permit the use of a large volume at one time. After irradiation the contents of six celluloid exposure cells (described below) were pooled and diluted to an arbitrary standard, using distilled water. The control suspensions were prepared by diluting a portion of the concentrated suspension in exactly the same ratio as the irradiated sample. Colorimetric measurements showed that the pH of the suspensions was approximately 6.2.

X-ray apparatus and technique. The source of the x-rays was a water cooled molybdenum target Coolidge tube mounted in the

General Electric x-ray diffraction apparatus, (Davey (1921 and 1922)). The tube was operated as a self-rectifying tube at 30 K.V._{R.M.S.} The discharge current varied from 35 to 45 milliamperes. This current was read every minute so that the total radiation dose, expressed as the product of discharge current times time, could be expressed in milliamperes-minutes at 30 K.V._{R.M.S.} at 15.2 cm. distance (Mo target). No filters were used.

Exposure cells of approximately 50 by 10 by 3 mm. were made by forming a box of celluloid about the end of a piece of glass. These cells were very transparent to the radiation, and the shape gave as great and as uniform exposure to this radiation as was practicable. When filled with the aqueous suspension of bacteria, the cells were mounted at the lower openings of the x-ray apparatus and the protective door was closed as far as possible. Each cell, therefore, was irradiated by the direct rays from the Coolidge tube. The x-ray dosage (see table 2) ranged from 245 to 3,000 Ma-M at 30 K.V._{R.M.S.} at 15.2 cm. distance (Mo target).

Measurements of electrophoretic migration velocity. The apparatus used was the capillary cell type described by Falk, *et al.* (1928). Since the migration velocity in this apparatus is dependent in part upon the bore and length of the capillary, preliminary tests were made upon a number of capillaries of uniform length. From these results several capillaries which gave almost uniform migration velocities were selected for use in this study. Having made such measurements on each capillary the later readings could be compared by simple proportion regardless of the individual capillary used. This precaution is quite essential because of the danger of breakage.

Measurements of migration velocities were made upon 10 bacterial cells (5 with each polarity of the electrical field). The capillary was then refilled and a second set of measurements was made. The two sets of measurements were averaged, provided their difference was not greater than 4 per cent. In case of greater differences further measurements were made. The results are expressed in terms of microns per second at 40 volts.

The capillaries and cell were cleaned thoroughly with dichromate-sulphuric cleaning solution and distilled water. Especial

attention is called to the need for absolute cleanliness of all glassware and the use of only high grade distilled water.

Determination of viability and pH. In a portion of the study, ordinary plate counts and colorimetric measurements (Lamotte

TABLE 1
Electrophoretic migration velocities of repeated experiments using various capillaries

CAPILLARY	DATE	VELOCITY <small>μ/sec.</small>	AVERAGE	PERCENTAGE DEVIATION FROM AVERAGE
1	January 28	27.17	27.21	-0.143
	January 29	26.99		-0.808
	January 30	27.12		-0.313
	February 26	27.57		+1.312
3	January 28	28.13	27.66	+1.699
	January 30	27.64		-0.072
	February 26	27.85		+0.687
	March 3	27.85		+0.687
	March 3	27.46		-0.723
	March 5	27.06		-2.133
5	January 28	26.92	26.80	+0.448
	January 30	26.68		-0.448
	January 31	26.80		0.000
	February 26	26.80		0.000
7	January 28	24.02	24.36	-1.395
	January 30	24.31		-0.246
	February 26	24.44		+0.328
	March 3	24.44		+0.328
	March 5	24.25		-0.451
	March 6	24.36		0.000
	March 7	24.36		0.000
	March 11	24.70		+1.395
	March 14	24.44		+0.328
	March 25	24.25		-0.451

standards) were made upon both the irradiated and control suspensions.

EXPERIMENTAL RESULTS

Experiments, made to determine the experimental error in the hands of the operator, showed that almost identical readings could

TABLE 2
Summary of electrophoretic measurements

IRRADIATION DOSES (Ma-M AT 30 K.V. R.M.S. AT 15.2 CM. DISTANCE (Mo TARGET))	NUMBER OF TESTS	MIGRATION VELOCITY	
		Rayed $\mu/sec.$	Control $\mu/sec.$
245-247	3	24.58	24.25
496-499	3	24.09	24.36
1,001-1,013	6	24.31	24.39
1,437	1	24.44	24.61
1,505-1,529	5	24.28	24.32
2,000-2,013	6	24.28	24.39
2,541-2,555	4	24.39	24.44
3,000-3,009	3	24.36	24.45

TABLE 3
Results of viability studies

IRRADIATION DOSES (Ma-M AT 30 K.V. R.M.S. AT 15.2 CM. DISTANCE (Mo TARGET))	NUMBER OF VIABLE CELLS PER CUBIC CENTIMETER	
	Rayed	Control
1,001	2,100,000	2,130,000
1,011	2,000,000	1,760,000
1,505	2,430,000	2,130,000
1,514	1,790,000	1,930,000
2,000	2,270,000	1,930,000
2,013	2,580,000	2,710,000
2,550	2,660,000	2,710,000
3,000	1,410,000	1,350,000
3,003	2,890,000	2,710,000

TABLE 4
Results of pH determinations

IRRADIATION DOSES (Ma-M AT 30 K.V. R.M.S. AT 15.2 CM. DISTANCE (Mo TARGET))	pH		
	Distilled water	Suspensions	
		Rayed	Control
1,001	6.2	6.2	6.2
1,514	6.2	6.2	6.2
1,514	6.4	6.4	6.4
2,000	6.2	6.2	6.2
2,013	6.2	6.2	6.2
2,555	6.6	6.8	6.6
3,000	6.0	6.2	6.2

be obtained for each individual capillary when suspensions of the culture were prepared upon different days as indicated in table 1. An inspection of the data shows that the readings with a single capillary did not vary more than 4 per cent, thus confirming the accuracy of the method as reported by Falk, *et al.* (1928), Chapman (1929) and in previous unpublished studies by ourselves.

The results of electrophoretic migration velocities (table 2), plate counts (table 3) and pH determinations (table 4), all show very clearly that the x-rays of the wave lengths studied did not alter the electrophoretic charge, viability or pH of *Esch. coli* suspended in distilled water of pH 6.2. Macroscopical examination of the irradiated suspensions revealed no evidence of lysis and plate counts (table 3) showed no lytic effect upon the living organisms. These results are quite different from those obtained with ultraviolet irradiation, Lisse and Tittler (1931). In that case there was a marked change in electrophoretic velocity, a decided lethal effect, lysis and increase in the pH of the suspension.

The consistent electrophoretic velocities of controls (table 2) are in harmony with unpublished data obtained during two years and also with the recent report by Chapman (1929).

DISCUSSION

The changes in the electrophoretic migration velocity which accompanied the lethal effect of ultraviolet radiations suggested that if x-rays kill bacteria they should also produce changes in the migration velocity. On the contrary, if x-rays do not affect the electrophoretic migration velocity, no injurious or lethal effects should be expected in view of our hypothesis that injury and death are accompanied by changes in the migration velocity. The latter assumption is supported by the results of this investigation which show very clearly that neither migration velocity nor viability were affected. This does not exclude the possibility that these rays may produce changes in the migration velocity under experimental conditions which kill bacteria.

The fact that many investigators found injurious or lethal effects produced by x-rays while others, including ourselves, failed to detect any such effects indicates differences in experi-

mental conditions. The literature shows that both biological and physical conditions have varied considerably from one investigation to another. The cultures used may have varied in sensitivity due to differences in both the nutritive medium and the period of incubation. The environmental conditions under which the bacteria were irradiated have also differed greatly since broth cultures, freshly inoculated agar plates and saline or water suspensions have been used. Undoubtedly the differences in quality and quantity of x-rays used have contributed greatly to the differences in the effects. However, in many instances little if any information was given concerning either the quality or intensity of rays emitted. Such variations render a comparison of the various investigations impossible. In view of the differences between the experimental conditions of the various investigations it is not surprising that different results have been obtained.

Using Mo rays at 34 to 38 K.V._{R.M.S.}, Wyckoff (1930b) found that a measureable proportion of *B. coli* was killed by doses of about one one-hundredth the amount which we found ineffective. Apparently there were only two important differences between his experimental conditions and ours. First, he irradiated organisms immediately after they were spread on the surface of agar, while we irradiated an aqueous suspension. Secondly, he used an x-ray tube having a Lindemann window which transmitted not only characteristic Mo-K rays but also very long and easily absorbed rays, while we used an x-ray tube made of the commercial type of glass. Even if the glass of our tube had permitted the passage of the extremely long rays they would have been absorbed by the first layers of the water suspension and therefore rendered practically ineffective. Wyckoff obtained very similar results with silver, copper, chromium and molybdenum targets in the x-ray tube, and the use of an Ag target gave almost identical results in the same time of exposure irrespective of whether he used a voltage of 21 K.V._{R.M.S.} (giving Ag-L rays in addition to the continuous spectrum) or a voltage of 34 K.V._{R.M.S.} (giving Ag-K rays in addition to the continuous spectrum). Therefore, we are tempted to assume that the Ag-K and Ag-L rays were no more effective than our Mo-K rays and that the lethal effect was

due entirely to the long wave lengths of the continuous spectrum transmitted by the Lindemann window and absorbed by the bacteria on the agar surface.

There is also the possibility that the agar was influenced by the radiation in such a manner that the bacteria were affected by some secondary action which was not produced in our aqueous suspension. We are inclined to discount this possibility but do not believe that it can be entirely discarded. Further experiments are contemplated to test this point.

SUMMARY

1. The effects of "low voltage" x-rays (35 K.V._{R.M.S.}) upon bacteria are discussed on the basis of a theory relating stimulation, injury and death to changes in the electrophoretic migration velocity.

2. During this and other investigations, the electrophoretic migration velocity of *Esch. coli* remained constant for over two years.

3. Using the total radiation from a Coolidge x-ray tube at 30 K.V._{R.M.S.} (without filters) no changes were detected in the electrophoretic migration velocity, viability or pH of an aqueous suspension of *Esch. coli* with exposures as great as 3000 milli-ampere-minutes at 15.2 cm. target-culture distance. This dose is about 100 times as great as that for which Wyckoff found a lethal effect. Possible causes for the difference in results have been discussed.

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tion of organic chemicals" contains directions for preparing the following compounds: acetyl benzoin, *d*-arginine hydrochloride, benzyl phthalimide, *p*-chlorobenzaldehyde, desoxybenzoin, desyl chloride, dibenzalacetone, 1,2-dibromocyclohexane, 2,6-diiodo-*p*-nitroaniline, 2,4-dinitrobenzaldehyde, 4,5-diphenylglyoxalone, ethyl α,β -dibromo- β -phenylpropionate, ethyl-*N*-methylcarbamate, hippuric acid, iodothiophene, mercury di- β -naphthyl, methyl isopropyl carbinol, *S*-methyl isothiourca sulfate, β -naphthylmercuric chloride, nitrobarbituric acid, phenylpropionic acid, phenyl thienyl ketone, propionaldehyde, succinic anhydride, β -thiodiglycol, thiophene, thiosalicylic acid, *p*-tolualdehyde, uramil, and diethyl zinc.

The subject index includes also the material in Volumes X and XI, volumes which are not combined in the collective work which appeared recently. [For reviews of Vols. X, XI, and the Collective Volume, respectively, see *J. CHEM. EDUC.*, **7**, 1218 (May, 1930); **8**, 1242 (June, 1931); **9**, 1499 (Aug., 1932).] Later references to certain of the preparations described in these same two volumes are also included.

The arrangement of subject matter is the customary one: a section which describes procedure is followed by a second which includes notes on specific parts of the procedure. A third section gives a very brief summary of other methods of preparing the same substance, and includes literature references.

All of the methods described have been tested in a laboratory other than that of the contributor, so that one can depend on obtaining the described results.

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Chemistry of Food and Nutrition. HENRY C. SHERMAN, Ph.D., Sc.D., Mitchell Professor of Chemistry, Columbia University. Fourth edition. The Macmillan Co., New York City, 1932. xiii + 614 pp. 32 Figs. 13 × 20 cm. \$3.00.

The author states that "the purpose of this book is to present the principles of the chemistry of food and nutrition both as an integral part of the study of chemistry and with reference to the food requirements of man and the considerations which should underlie our judgment of the nutritive value of foods and the choice and use of food for the maintenance and advancement of positive health and vitality." That the author has accomplished this purpose is probably best indicated by the favorable reception which previous editions of the book have been accorded by teachers and students of nutrition.

The present book is rewritten and enlarged but follows the same general plan which characterized previous editions. A comparison of the third and fourth editions shows that the latter contains twenty-four chapters, while the former consisted of but twenty. The former brief "Introduction" has been expanded into Chapter I of the new edition. Formerly the author discussed proteins in a single chapter. In the new edition one chapter is devoted to "The General Chemistry of the Proteins and Amino Acids" and a new chapter has been added expanding the "Nutritional Chemistry of the Proteins and Amino Acids." The chapter on "Iron in Food and Nutrition" is now "Iron and Copper in Food and Nutrition" and covers the recent work on iron and copper in blood regeneration.

The reviewer feels that the new edition is materially strengthened by the addition of a new chapter entitled "Acid Base Balance in Foods and Nutrition," in which the author has brought together into one chapter the discussion of hydrogen-ion concentration, buffer action, blood buffers, acid elimination, effect of diet, etc. From a pedagogical point of view this is often much more effective than to discuss the various chemical and biological phases of acidity and alkalinity at scattered points throughout the text.

It is for this reason that the reviewer regrets to find that an important former chapter entitled "Chemical Nature and

Regulation of Oxidative Processes in the Body" had been omitted in the new edition. The probable mechanism of biological oxidations is a matter of extreme interest to students of biology, medicine, and nutrition and, in the mind of the reviewer, is sufficiently important to be treated as a special topic.

The vitamin chapters have been expanded to include separate chapters for vitamins B and G. The appendixes have been modified and some new additions have been made to this very useful portion of the book. Carefully selected and revised bibliographies are appended to each chapter.

The index, which occupies 39 pages, is unusually complete but has one fault which is likewise common to previous editions, *viz.*, that a single topic may be followed by as many as 50 page citations. Considerable time could be saved for the busy reader if this fault could be remedied in future editions.

No teacher whose work involves the application of chemistry to biological problems can afford to call his active reference list complete unless Sherman's "Chemistry of Food and Nutrition" is included. The choice of subject matter, the clarity with which involved scientific topics are treated, and the authoritative but fair and impartial way in which the author, in his characteristic manner, discusses controversial theories, must command the reader's interest and admiration.

Dr. Sherman's new text will commend itself to every teacher and student who desires a well-printed text containing a wealth of chemical and nutritional information interpreted by an outstanding teacher and research worker.

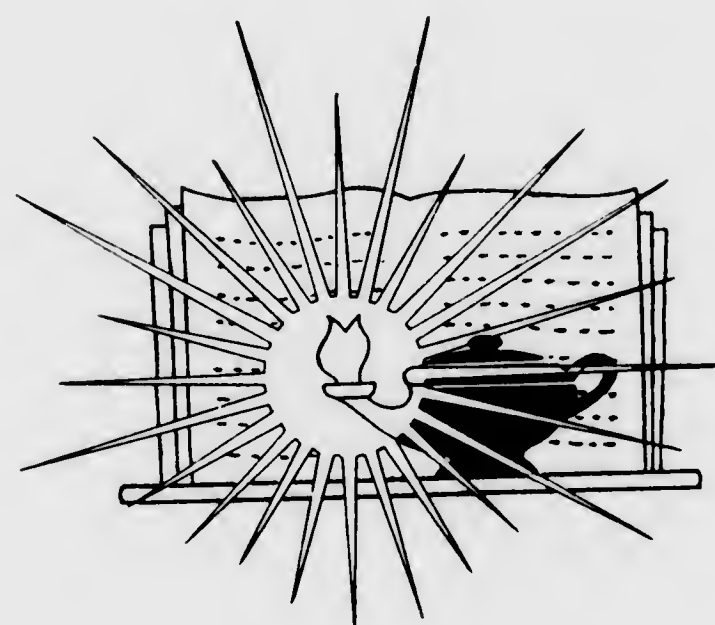
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MISCELLANEOUS PUBLICATIONS

Education in Belgium. JAMES F. ABEL, Chief, Division of Foreign Schools Systems. U. S. Dept. Interior, Office of Education, Bulletin, 1932, No. 5. U. S. Government Printing Office, Washington, D. C. (For sale by the Superintendent of Documents, Washington, D. C.) 145 pp. 15 × 23 cm. \$0.15.

Science Reading Material for Pupils and Teachers. C. M. PRUITT. Reprint from *Science Education*, Montclair, N. J., 1932. 27 pp. 15.5 × 23.5 cm. \$0.20.

While this suggestive reading list covers the whole field of science, it is classified under various headings so that the chemistry teacher may easily pick out the lists of particular interest to him. Pupil references, teacher references, and periodical literature appear in separate lists. Representative chemistry textbooks, both college and high-school, workbooks, and tests are included.



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Vol. XCVIII, No. 1, October, 1932

THE ASSAY OF VITAMINS B AND G AS INFLUENCED BY COPROPHAGY*

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(Received for publication, July 19, 1932)

When autoclaved yeast is used as the source of vitamin G for supplementing a vitamin B complex-deficient diet, a lack of uniformity in the response of our experimental animals has often been noted. Some rats died within a period of 15 to 20 days without manifesting the characteristic symptoms of vitamin B deficiency until the time of death. Other animals grew at a slow rate for 6 to 9 weeks, at which time typical paralyses occurred and persisted for several days. In a number of cases we have been unable to obtain paralytic symptoms in a period of 20 to 24 weeks. These animals usually grew at a fairly uniform but subnormal rate.

The cause of these irregularities could not be traced to variations in initial weights of animals or to litter variations. It was found, however, that the difficulty could be eliminated almost completely if larger meshed screens (two meshes to the inch) were used, thereby facilitating the passage of fecal particles through the screen. We have noted that animals receiving a vitamin B-deficient diet tended to be much more coprophagous than those which are fed a diet adequate in vitamin B but deficient in vitamin G. These and other observations suggested the necessity for further investigations relative to the causes of the above irregularities.

Sufficient experimental evidence (1-22) has been published to demonstrate that the vitamin B complex can be synthesized by microorganisms and that coprophagy may affect growth response to a marked degree.

* Published by permission of the Director of the Pennsylvania Agricultural Experiment Station as technical paper No. 562, Journal series.

The experiments described in the present paper were conducted to determine the extent to which rats can synthesize vitamin B or vitamin G when the experimental diet is deficient in one or both of these factors.

EXPERIMENTAL

The basal diet consisted (in parts per 100) of washed casein 18, salt mixture (McCormick's 185 (23)) 4, agar 2, sucrose 15, dextrin 56, cod liver oil 2, and filtered butter fat 3. All the ingredients, with the exception of the cod liver oil and butter fat, were pulverized, mixed thoroughly, heated for 10 hours at 100–105° and stored in glass containers. The cod liver oil and butter fat were kept in a refrigerator and added to the basal diet, in the proportions indicated, just prior to the time of feeding.

The vitamin B supplement was prepared by percolating 95 per cent ethyl alcohol through dried brewers' yeast until the alcohol came through devoid of color. The percolate was concentrated under diminished pressure until the residue assumed a semisolid consistency. The residue was dried in a vacuum over sulfuric acid, macerated with cold 95 per cent alcohol, and filtered. The filtrate was made up to such a volume that 0.1 ml. of the solution represented 1 gm. of the original yeast.

The vitamin G supplement was made from bakers' yeast by adding ethyl alcohol to the aqueous extract of the yeast until the alcoholic concentration reached 50 per cent by volume. The precipitate obtained at this point was discarded, and the concentration of alcohol was increased to 80 per cent. A second precipitate was obtained, which was removed by filtration, dried, autoclaved for 6 hours at 15 pounds pressure, again dried, and pulverized. To facilitate the feeding of this fraction, it was diluted with powdered dextrin so that 0.3 gm. of the mixture represented 1 gm. of the original yeast.

Piebald rats, 20 to 21 days of age and weighing from 39 to 45 gm., were placed in individual cages provided, during the depletion period, with galvanized bottoms or grids which contained three meshes to the inch. At the end of the depletion period these screens were replaced by grids containing but two meshes to the inch. The cage pans were equipped with absorbent paper which absorbed the urine and facilitated the cleaning of the pans.

The animals were weighed at intervals of 1 week, at which times records were made concerning food consumption and physical condition. The time required for cessation of growth ranged from 10 to 24 days. At this point the animals were assigned to the various experimental groups, care being taken to observe the usual precautions regarding sex and litter origin.

TABLE I
Effect of Vitamin B and Vitamin G Supplements on Rats of Series 1 with Small Meshed Screens Used during Depletion Period

Group No.	No. of rats per group	Vitamin B concentrate fed daily	Vitamin G concentrate fed daily	Feces fed daily from group No.	Average initial weight	Weight at end of depletion period	Average gain in weight	Average weekly food intake	Rats surviving at end of experiment
		ml.	gm.		gm.	gm.	gm.	gm.	
1	4	0.1	0.3		40	47	93	39	4
2	4		0.3	8	42	58	78	37	4
3	4	0.1		8	41	48	73	38	4
4	4		0.3	9	41	54	69	39	4
5	4	0.1		9	40	56	66	37	4
6	4			9	40	47	45	28	4
7	4			8	42	51	38	26	4
8	12	0.1			40	46	19	28	12
9	16	*	0.3		41	49	15	30	14
10	4				41	50	0	12	0†

* Each rat of this group received, weekly, 0.1 ml. of the vitamin B concentrate to prevent death from beriberi.

† All rats of this group died between the 27th and the 49th day after being placed on the basal diet.

The first series of experiments consisted of ten groups of animals which were fed the various supplements to the basal diet according to the outline given in Table I.

The animals in Groups 8 and 9 were placed on experiment from 12 to 20 days earlier than those of Groups 2 to 7 in order that their feces might be available for study with the last mentioned groups. The feces from animals in Groups 8 and 9 were collected from one to three times a week and stored under ether until the end of each experimental week. At this time the feces were pulverized and washed four times with ether. After the retained ether had been allowed to evaporate the finely pulverized material was stored in

glass containers. The daily allotment per rat was equivalent to the average daily amount of feces voided per day during the corresponding experimental week by each animal in Groups 8 and 9. The average daily amount of excreta voided per rat varied from 0.17 to 0.28 gm. of dry extracted feces. To facilitate the weighing of the daily allotment, sufficient powdered dextrin was

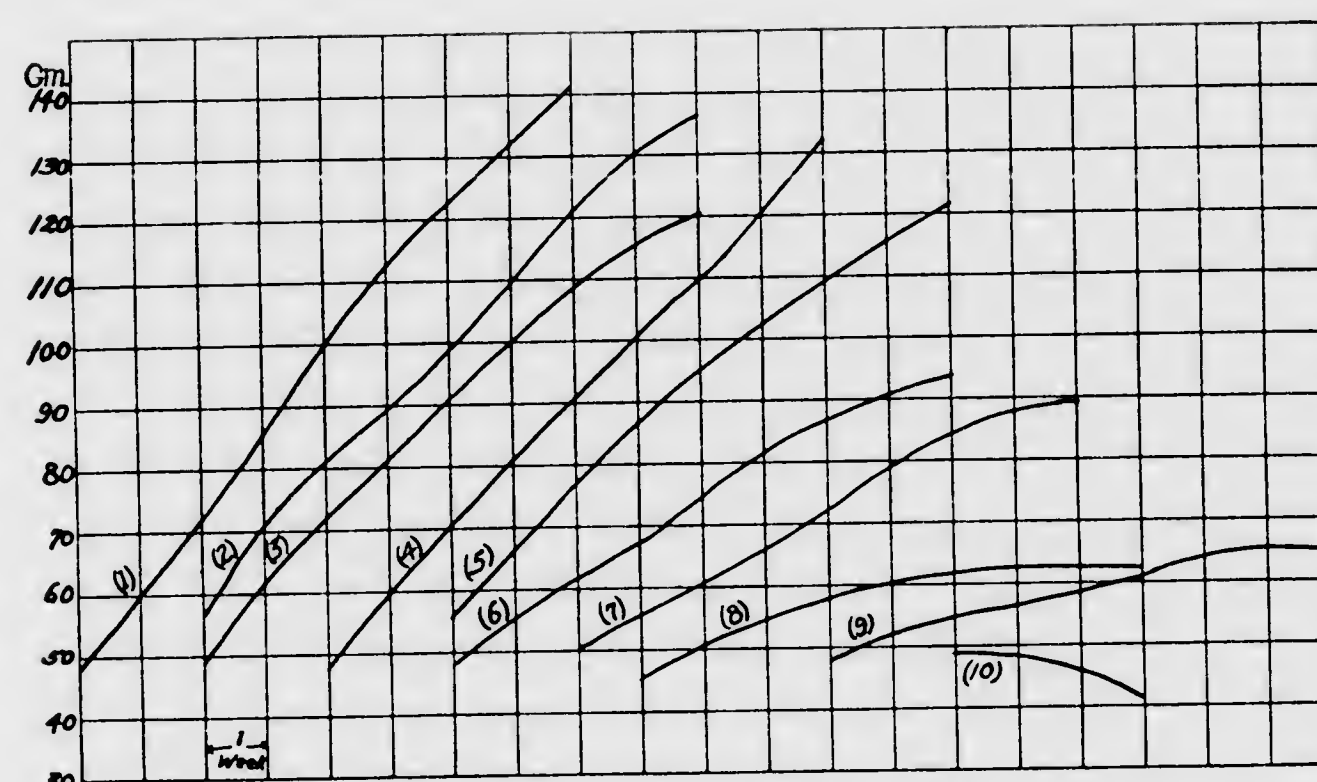


CHART 1. Growth response made by the several groups of rats constituting Series 1, with small meshed screens used during the depletion period. Group 1, basal diet + vitamins B and G; Group 2, basal diet + vitamin G + feces from Group 8; Group 3, basal diet + vitamin B + feces from Group 8; Group 4, basal diet + vitamin G + feces from Group 9; Group 5, basal diet + vitamin B + feces from Group 9; Group 6, basal diet + feces from Group 9; Group 7, basal diet + feces from Group 8; Group 8, basal diet + vitamin B; Group 9, basal diet + vitamin G; Group 10, basal diet alone.

added so that 0.3 gm. of the mixture represented the average daily elimination per rat during the preceding week.

The daily supplements were fed in special receptacles separate from the basal diet for a period of 8 weeks following the depletion period. The results obtained are given in Table I and Chart 1. Complete food intake records are omitted here, since food intake and growth response paralleled each other in a markedly uniform manner.

Examination of Chart 1 shows that the average body weights of the various groups at the beginning of the curative period were quite variable. This was due to the fact that the animals showed considerable variations in the time required for depletion. It was thought that these variations might be explained on the assumption that some coprophagy had occurred during the depletion period due to the small size of openings in the screens or grids. It was for this reason that the larger meshed screens were introduced

TABLE II
Effect of Vitamin B and Vitamin G Supplements on Rats of Series 2 with Large Meshed Screens Used during Entire Experiment.

Group No.	No. of rats per group	Vitamin B concentrate fed daily	Vitamin G concentrate fed daily	Feces fed daily from group No.	Average initial weight	Weight at end of depletion period	Average gain in weight	Average weekly food intake	Rats surviving at end of experiment
		ml.	gm.		gm.	gm.	gm.	gm.	
1	4	0.1	0.3		41	49	86	33	4
2	4		0.3	8	40	52	69	31	4
3	4	0.1		8	39	53	68	36	4
4	4		0.3	9	42	54	66	38	4
5	4	0.1		9	40	52	65	38	4
6	4			9	41	53	60	31	4
7	4			8	39	51	58	33	4
8	12	0.1			42	54	13	23	12
9	12	*	0.3		42	52	12	22	12
10	4				42	49	0	14	0†

* All rats of this group received just sufficient vitamin B concentrate to prevent death from beriberi during the experimental period.

† All rats of this group died between the 34th and the 43rd day after being placed on the basal diet.

at the beginning of the curative period in Series 1. In order to obviate the discrepancies mentioned above, a second series of experiments was conducted in which the large meshed screens (two meshes to the inch) were used throughout the entire experimental period.

The details of management, feeding, etc., for Series 2 were identical with those described for Series 1. At the end of the depletion period (which was much more uniform than that of Series 1) the animals were divided into ten groups, after the usual

precautions regarding litter and sex distribution were observed. The diet and supplements of the various groups were identical with those of Series 1, as is shown in Table II, with the exception of Group 9. Instead of administering a weekly supplement of vitamin B to each animal as in Series 1, the supplement was not added to the diet until the animals had shown definite symptoms

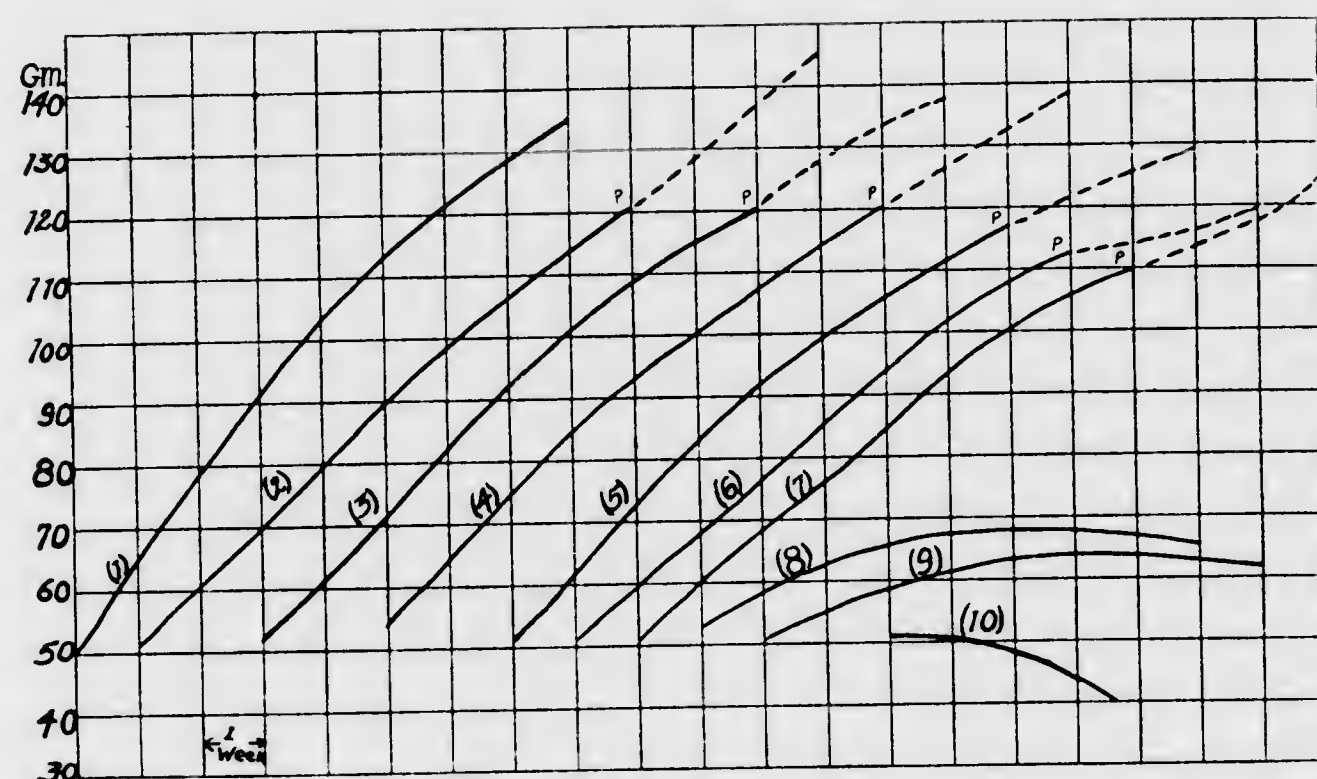


CHART 2. Growth response made by the several groups of rats constituting Series 2, with large meshed screens used during the entire experiment. Group 1, basal diet + vitamins B and G; Group 2, basal diet + vitamin G + feces from Group 8; Group 3, basal diet + vitamin B + feces from Group 8; Group 4, basal diet + vitamin G + feces from Group 9; Group 5, basal diet + vitamin B + feces from Group 9; Group 6, basal diet + feces from Group 9; Group 7, basal diet + feces from Group 8; Group 8, basal diet + vitamin B; Group 9, basal diet + vitamin G; Group 10, basal diet alone. P = changed to feces of rats receiving adequate amounts of vitamins B and G daily.

of vitamin B deficiency and the amount fed, from this point, was the minimum amount required to relieve paralytic symptoms and maintain life without producing a pronounced effect on growth.

The experiments in Series 2 differed in one other respect; *viz.*, after the usual 8 week curative period, Groups 2 to 7 received daily supplements of 0.3 gm. of extracted feces obtained from rapidly growing rats which had received daily allotments of dried bakers'

yeast. The data obtained by this change in feeding procedure are indicated in Chart 2 by the broken line extensions of the growth curves.

At the time these data were being assembled for publication, Roscoe's paper (24) appeared, emphasizing the significance and importance of coprophagy and refection in experimental rats. Our findings with few exceptions are in general agreement with those of Roscoe, although the methods of attack are somewhat different.

DISCUSSION

The marked similarity in results obtained in Series 1 and 2 (Charts 1 and 2) indicates that the variability in depletion time and in body weight at the beginning of the curative period in Series 1 had no appreciable effect in the subsequent deportment of the experimental animals. It would appear, therefore, that it is possible to discuss the results in terms of corresponding groups in both series of experiments.

The animals receiving the supplemented basal ration (Groups 10) responded in much the same manner, with the exception that those in Series 1 died between the 27th and 49th days, while those of Series 2 all died within a 9 day period, *viz.*, between the 34th and 43rd days of the experiment. Some of the animals manifested characteristic paralytic symptoms just prior to death, while others died without showing these symptoms. After death, however, the latter were found clutched, by mouth or by paws, to the side or bottom of the cage. The groups which received the vitamin G supplement (Groups 9) averaged 15 and 12 gm., respectively, in the 8 week period, and paralysis was observed quite frequently, especially in Series 2.

When vitamin B was the sole supplement to the basal ration (Groups 8) better initial growth was obtained, although there was a tendency for weight to decline toward the end of the 8 week experimental period. Many of these animals developed scaly feet and a mild form of dermatitis on the head and neck.

The groups which received the basal ration supplemented by feces from vitamin G-deficient animals (Groups 7) made an average gain of 5 and 7 gm. per week, respectively, and all animals remained in apparent good health until the experiment was ter-

minated. Our observations differed from those of Roscoe (24) in that we failed to note any abnormal size or volume of the feces other than that which could be explained on the basis of increased food intake. We noted that these animals tended to develop rough or shaggy fur.

The groups which received the basal ration supplemented by feces from vitamin B-deficient animals (Groups 6) grew at a rate quite comparable with that shown by Groups 7. Comparison of Curves 10 (Charts 1 and 2) shows quite clearly that diets deficient in vitamins B and G can be supplemented to advantage with feces. The addition of feces from normal rats receiving 0.6 gm. of yeast per day did not prove beneficial so far as stimulation of new or more vigorous growth was concerned.

Groups 5 which received the basal ration supplemented with the vitamin B concentrate and feces from Groups 9 showed an average weekly gain of about 8 gm. The response of Groups 4 was quite comparable with that of Groups 5.

The results obtained in Groups 3 show how feces from vitamin G-deficient rats can supplement a vitamin G-deficient diet. Groups 2 grew well on a diet deficient in vitamin B when the diet was supplemented by feces from rats which had received adequate amounts of this vitamin.

Our data are in general agreement with those of Roscoe (24) regarding the stimulatory effect of feces. The growth responses in Groups 2 to 7 show quite conclusively that vitamins B and G were supplied from feces in sufficient amount to produce satisfactory growth. We are unable to explain the method or mechanism by which vitamins B and G are synthesized. It is possible that feces contain a substance or substances which encourage the growth of bacteria in the intestine. This has been suggested by Roscoe (24). Kennedy and Palmer (18) believe that the stimulatory effect induced by coprophagy is due to the presence of an unknown vitamin or substance which stimulates growth of the rat. Roscoe states that sterilization of feces did not destroy the growth-promoting properties. If this is true it would appear that growth stimulation cannot be due to reinfection. Since all existing data indicate that body storage of the vitamin B complex is quite limited, we are forced to conclude that the beneficial effect of coprophagy is not due solely to the excretion of the original body

stores of vitamins B and G as was indicated in a previous publication from this laboratory (8).

The responses obtained in Groups 3 and 4 indicate quite clearly that both vitamins are synthesized in the body. This is borne out by the fact that good growth was obtained when feces from vitamin B- and G-deficient animals were used to supplement vitamin B- and G-deficient diets. Our data seem to indicate that the relative quantities of these vitamins eliminated in feces are approximately equal and that the amount eliminated is independent of the limiting factor in the diet. A number of observations have been made which show that animals receiving the basal diet without supplement, eliminate feces which are potent in both factors.

Four male rats (Rats 6479, 6480, 6481, and 6482), all from the same litter, were placed in the usual type of cages and were fed the basal diet. The records of their dietary régime and responses are recorded here, since they are not included in Tables I and II and Charts 1 and 2.

Rat 6479 weighed 45 gm. when placed on the basal diet and reached its maximum weight of 64 gm. by the 14th day. From this point the weight of the animal gradually decreased, with symptoms of beriberi appearing on the 35th day and death occurring on the 43rd day.

Rat 6480 weighed 42 gm. when placed on the basal diet and gained 10 gm. during the first 14 days. On the 28th day it manifested symptoms of beriberi and was given daily, during the next 14 days, its own feces eliminated during the previous 24 hours. Its weight increased from 48 to 67 gm. during this time. The feces feeding was discontinued during the following 14 days and the weight of the animal decreased to 50 gm., without any apparent symptoms of beriberi being observed. The feces were again fed for 14 days, as previously indicated, and the weight increased to 85 gm.

Rat 6481 weighed 43 gm. when placed on the basal diet and attained a weight of 52 gm. by the 14th day, at which time the weight began to decline. On the 29th day the animal weighed 46 gm. and showed marked symptoms of beriberi. It was given its own feces for 14 days. During this time the paralytic symptoms disappeared and the weight increased to 77 gm. The feces feeding

was discontinued from this point and body weight decreased gradually until death resulted on the 77th day. At this time the animal weighed 40 gm. and had manifested slight symptoms of beriberi.

Rat 6482 weighed 40 gm. when placed on the basal diet and made a 9 gm. increase during the first 14 days on this diet. Paralytic symptoms were noted on the 28th day, at which time the animal was fed its own feces for the next 14 days. During the 14 days in which the feces supplement was fed, the paralytic symptoms disappeared and body weight increased from 42 to 57 gm. The feces supplement was then omitted from the diet and the weight of the animal decreased gradually until the 65th day, when death occurred. At the time of death the animal weighed 36 gm.

Thus, as a result of receiving its own feces as a supplement to a vitamin B complex-deficient diet, each of three animals showed a definite increase in weight and a marked improvement in physical condition, while their litter mate, which did not receive the supplement, grew gradually weaker and died at an earlier date.

While our studies do not answer the question as to the mechanism of growth stimulation in coprophagous rats, they seem to show that studies involving the assay of foods for vitamins B and G can be vitiated by the introduction of adequate amounts of these vitamins if coprophagy is permitted. Coprophagy can be reduced to a minimum if screens with large meshes are used. Accurate experiments involving assays for vitamins B and G may necessitate a series of screens of different sized meshes to be changed from time to time as the animals increase in size. The question of coprophagy still remains a serious problem and merits continued study. We hope to continue the study with the view of investigating the bacteriological phases of the problem.

SUMMARY

1. A quantitative study is described in which rats were fed diets deficient in vitamins B and G, respectively. Feces from these rats were fed to other rats as supplements to diets which were also deficient in these vitamins.
2. Rats which received diets deficient in vitamins B or G eliminated feces which were potent sources of these vitamins.
3. The potency of the feces eliminated by vitamin B- and vita-

min G-deficient rats showed no tendency to become less potent as the experiments progressed.

4. The amounts of vitamins B and G found in feces seem to be about equal and do not seem to depend on the diet of the animal.

5. No satisfactory explanation can be furnished at the present time regarding the mechanism whereby these vitamins are synthesized.

6. The danger of coprophagy, as it affects the accuracy of assays of vitamins B and G, is discussed.

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A BIOCHEMICAL STUDY OF IRRADIATED MILK

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Recently O'Brien, Davis and Armstrong¹ have described a method for irradiating milk with ultraviolet light. Their source of light is an arc from carbon electrodes of special composition and has an energy emission, in the wave length region from 2,800 to 3,100 Ångstrom units, 1,260 times that of bright sunlight. There is very little energy emitted at wave lengths shorter than 2,800 Å. They point out that radiation of wave lengths between 2,800 to 3,100 Å. is very effective in producing anti-rachitic potency, while radiation of wave lengths less than 2,800 Å. is destructive to vitamin A, causes protein coagulation and produces a bad odor and taste in the milk. The apparatus in which the milk is irradiated is essentially a glass cylinder. The carbon arc is in the center of this cylinder and the milk flows down the inside of the cylinder in a thin film in such a way that each particle of milk receives a uniform dosage of light. Feeding experiments which they have conducted show that the vitamin D potency of irradiated milk is increased 16 times, while the vitamin A potency is 86 per cent. of that in the original milk. They also report bacteriological studies showing a decided reduction in the bacterial count after irradiation.

In the present paper a biochemical study of milk and butter from milk irradiated by the above process is reported. Since it was anticipated that effects of irradiation would be slight, the milk used in the following work was given a much longer exposure to ultraviolet light than is the usual practice. This was accomplished by passing it thru the irradiating process eight times. In this way it was hoped that any effects of ultraviolet light would be greatly exaggerated and therefore more easily detected by the methods employed. The samples of certified milk were irradiated at a plant located near Erie, Pennsylvania. Uniform samples of irradiated and non-irradiated milk were cooled, placed in thermos bottles and shipped to our laboratory at State College, arriving the following morning in good condition.

EXPERIMENTAL

Chemical Composition of Milk

The object of this phase of the work was to determine what changes in chemical composition, if any, take place during irradiation. Several sets

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¹ O'Brien, B., Davis, A. G., and Armstrong, E. L. Combined bactericidal and anti-rachitic effects induced in milk by carbon arc irradiation. Penna. Med. Jour. April, 1932.

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Recently O'Brien, Davis and Armstrong¹ have described a method for irradiating milk with ultraviolet light. Their source of light is an arc from carbon electrodes of special composition and has an energy emission, in the wave length region from 2,800 to 3,100 Ångstrom units, 1,260 times that of bright sunlight. There is very little energy emitted at wave lengths shorter than 2,800 Å. They point out that radiation of wave lengths between 2,800 to 3,100 Å. is very effective in producing anti-rachitic potency, while radiation of wave lengths less than 2,800 Å. is destructive to vitamin A, causes protein coagulation and produces a bad odor and taste in the milk. The apparatus in which the milk is irradiated is essentially a glass cylinder. The carbon arc is in the center of this cylinder and the milk flows down the inside of the cylinder in a thin film in such a way that each particle of milk receives a uniform dosage of light. Feeding experiments which they have conducted show that the vitamin D potency of irradiated milk is increased 16 times, while the vitamin A potency is 86 per cent. of that in the original milk. They also report bacteriological studies showing a decided reduction in the bacterial count after irradiation.

In the present paper a biochemical study of milk and butter from milk irradiated by the above process is reported. Since it was anticipated that effects of irradiation would be slight, the milk used in the following work was given a much longer exposure to ultraviolet light than is the usual practice. This was accomplished by passing it thru the irradiating process eight times. In this way it was hoped that any effects of ultraviolet light would be greatly exaggerated and therefore more easily detected by the methods employed. The samples of certified milk were irradiated at a plant located near Erie, Pennsylvania. Uniform samples of irradiated and non-irradiated milk were cooled, placed in thermos bottles and shipped to our laboratory at State College, arriving the following morning in good condition.

EXPERIMENTAL

Chemical Composition of Milk

The object of this phase of the work was to determine what changes in chemical composition, if any, take place during irradiation. Several sets

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¹O'Brien, B., Davis, A. G., and Armstrong, E. L. Combined bactericidal and antirachitic effects induced in milk by carbon arc irradiation. Penna. Med. Jour. April, 1931.

of samples were analyzed before and after irradiation. A typical set of analyses is given in table 1. The results are averages of three determinations.

TABLE 1
Showing the Analysis of Milk before and after Irradiation

	BEFORE IRRADIATION	AFTER IRRADIATION
Specific Gravity at 25° C.	1.0271	1.0272
Specific Conductivity	0.0049	0.0046
pH	6.57	6.74
Total Solids	13.28	13.22
Ash	0.69	0.69
Fat	4.40	4.31
Total Protein (N × 6.38)	3.48	3.42
Lactose	4.63	4.62
Sum (Ash, fat, protein, lactose)	13.20	13.04
Casein	2.50	2.47
Albumin	0.73	0.69

It will be seen that there are only slight differences in composition between the two samples. The pH of the irradiated sample is slightly higher than that of the non-irradiated sample. This can be accounted for on the basis of the reduction in bacterial count of the irradiated sample. Since the samples were not analyzed until the day following irradiation, more acidity may have developed in the untreated sample due to its higher bacterial count. Since ultraviolet light is known to cause a coagulation of albumin it was thought that possibly the albumin content of the irradiated sample would be considerably less than that of the untreated sample. The slight difference noted is hardly sufficient to be significant. Some of the difference can be accounted for by the lower total solid and protein content of the irradiated sample. Since the irradiated sample was irradiated eight times, it is certainly safe to say that normal irradiation by the process described does not cause detectable coagulation of the albumin in milk.

Chemical Composition of Butter Fat

In this work two samples of butter were used, one from untreated milk and the other from irradiated milk. The butter samples, of about a pound each, were melted and the butter fat used for the determination of various fat constants. Table 2 gives the results of the analyses. The results are averages of three determinations.

The most significant thing in table 2 is the shorter induction period for oxidation of the butter fat which had been irradiated. Irradiation undoubtedly initiates the oxidation process of the milk fat. It should be pointed out, however, that even after irradiation the sample studied had excellent

TABLE 2
Showing the Analysis of Butter Fat prepared from Milk before and after Irradiation

	BEFORE IRRADIATION	AFTER IRRADIATION
Refractive Index at 40° C.	1.4551	1.4551
Acid Number	0.93	0.96
Saponification Number	224.5	225.4
Acetyl Number	4.81	4.69
Reichert-Meissl Number	28.94	29.64
Polenske Number	1.51	1.89
Iodine Number	37.6	38.3
Kriest Test	Negative	Negative
Induction Period for Oxidation	20 hrs.	17 hrs.
Induction Period after 6 months storage	10 hrs.	8 hrs.

keeping qualities, superior to that of fat obtained from high quality creamery butter which gave an induction period for oxidation of 11 hours. In order to study the effect of irradiation on the keeping quality of butter fat the samples were stored in a refrigerator for six months and the induction period for oxidation determined again. It will be noticed that the induction period in both cases has been reduced about one-half. In other words the rate of deterioration is about the same in both cases.

In considering the fat constants it will be noticed that the changes after irradiation are slight. In every case except the iodine number the results lend themselves to the interpretation that slight oxidation has occurred. The higher acid, saponification, Reichert-Meissl and Polenske numbers of the irradiated sample, indicate a possible cleavage of long chain into short chain fatty acids. The lower acetyl number of the irradiated sample suggests the oxidation of hydroxyl groups. Since it is generally thought that oxidation takes place at the double bonds, one would expect a lower iodine number after irradiation. This is not the case. The higher iodine number after irradiation agrees well with the shorter induction period of this sample since unsaturated fats are known to have a shorter induction period than saturated fats. It is possible that irradiation shortens the induction period due to the introduction of double bonds in the molecule which is possibly a preliminary stage in the oxidation of a fat. Since such slight changes were found in fat exposed to such excessive doses of ultraviolet light, one can conclude that the usual dose would have an insignificant effect on the fat of milk.

Enzyme Studies with Pepsin

The object of this phase of the work was to determine what effect irradiation has on the rate of digestion of the proteins of milk by pepsin. The method used was as follows: 10 cc. of milk were placed in a beaker together

with 50 cc. of water and 5 cc. of 1.0 per cent commercial pepsin solution. This was then titrated with standard HCl solution to a pH of 2.0 at 25° C. using the quinhydrone electrode. This titration was made for both the irradiated milk and the control of non-irradiated milk. Next 10 cc. portions of each milk were placed in small Erlenmeyer flasks together with 50 cc. of water. The predetermined amount of standard HCl solution was then added to each flask to bring each solution to a pH of 2.0 after the addition of pepsin. These flasks were then placed in an incubator at 38° C. and allowed to remain there until they had reached a temperature of 38° C. Then 5 cc. of a 1.0 per cent commercial pepsin solution, which had a temperature of 38° C., were added to each flask. The flasks were kept at 38° C. throughout the experiment. At intervals two flasks of irradiated and of non-irradiated milk were removed and treated as follows: the samples were transferred to 200 cc. volumetric flasks and sufficient standard NaOH solution added to neutralize the added HCl solution and sufficient excess to make 14 cc. of N/10 NaOH in each flask. Then 4 cc. of Fehling's CuSO_4 solution were added to each flask and the solutions made up to volume. After a thorough mixing the solutions were filtered and 100 cc. portions of each filtrate were analyzed for nitrogen by the Kjeldahl method. The results indicate the amount of non-protein nitrogen present and are a measure of the amount of digestion taking place.

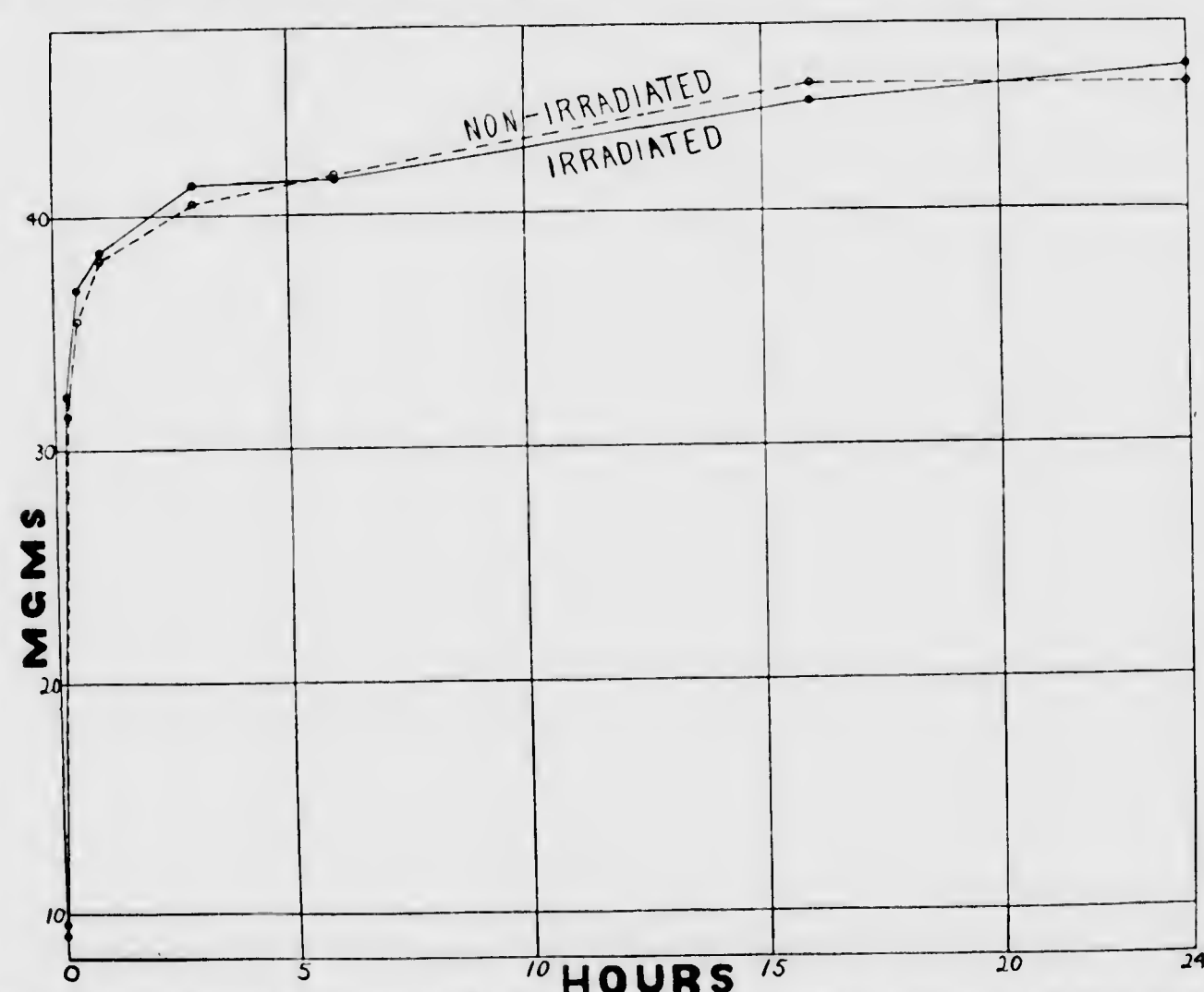


FIG. 1. SHOWING MILLIGRAMS OF NON-PROTEIN NITROGEN IN 10 CC. OF MILK DURING DIGESTION BY PEPSIN AT PH 2.0.

The above experiment was run several times and a typical set of results is given in Fig. 1. Each value is an average of duplicate determinations.

It should be noted that at the beginning of the experiment there was 0.5 mgm. more of non-protein nitrogen in the non-irradiated sample than in the irradiated sample. Hence, one should add 0.5 mgm. to each of the values in the curve for irradiated milk when comparing the two curves. If this were done the irradiated sample would show a greater amount of non-protein nitrogen at each period of analysis except the 16-hour period. Although the differences are not great, it appears that irradiation does not retard the digestion of milk proteins by pepsin but accelerates it slightly, especially during the early stages of digestion. Since the irradiated sample was given a heavy dose of ultraviolet light in this experiment as in the other experiments, it is very likely that normal irradiation by the process described has little effect on the digestibility of milk proteins by pepsin at a pH of 2.0.

Enzyme Studies with Trypsin

This phase of the work was very similar to that of the work with pepsin. The procedure differed in that the samples were brought to a pH of 8.1 with standard NaOH solution and at the end of digestion periods sufficient NaOH solution was added to make the total added equivalent to 14 cc. of N/10 NaOH. The trypsin used was a commercial preparation and 5 cc. of a 0.2 per cent solution were used in each flask.

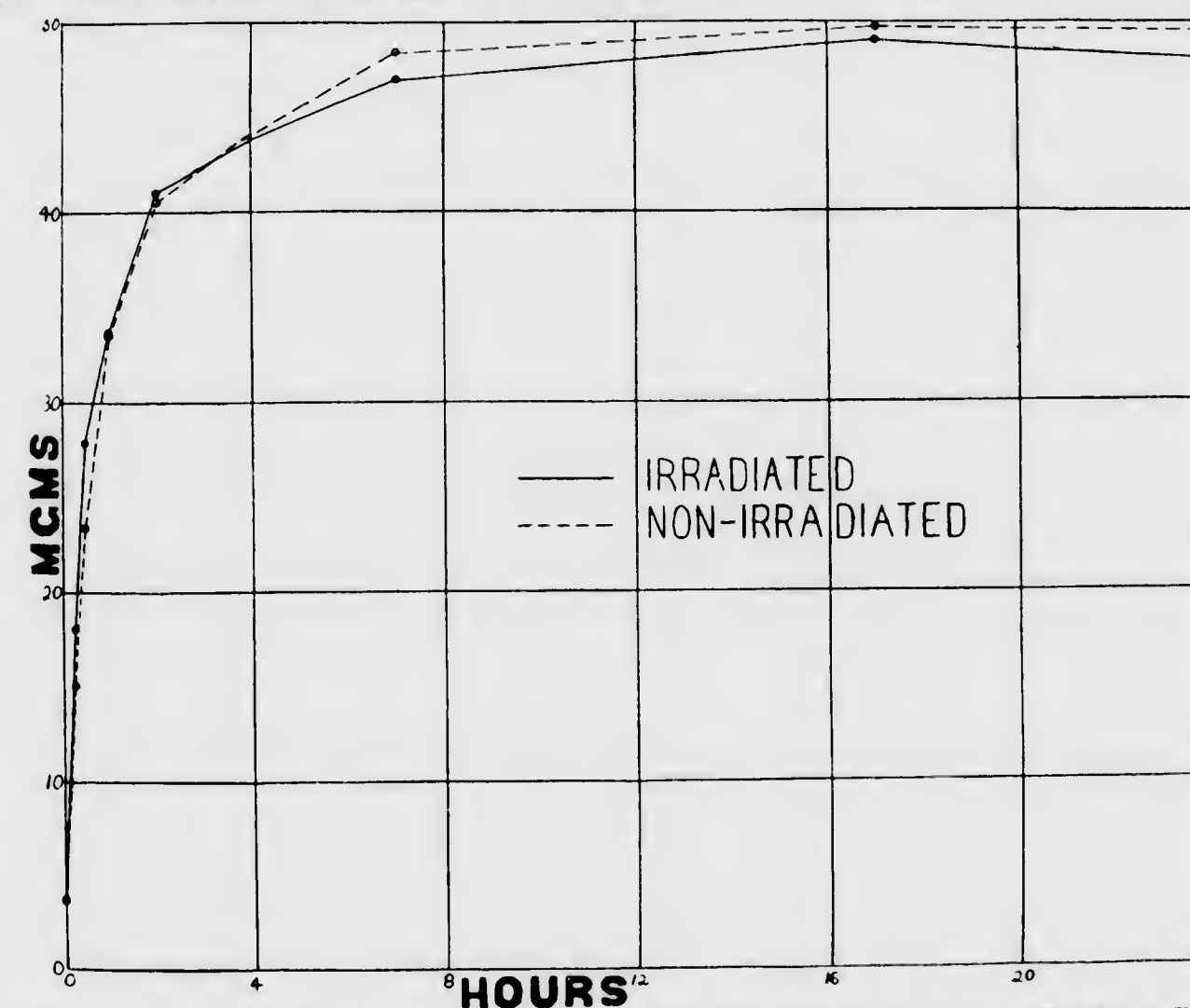


FIG. 2. SHOWING MILLIGRAMS OF NON-PROTEIN NITROGEN IN 10 CC. OF MILK DURING DIGESTION BY TRYPSIN AT PH 8.1.

Several experiments were run with trypsin, the results of a typical experiment being given in Fig. 2. The results are averages of duplicate determination.

The results of the work with trypsin are very similar to that with pepsin. The irradiated sample digests more rapidly during the early stages of digestion but finally after 4 hours the non-irradiated sample surpasses it. Here again it should be said that with normal irradiation by the method describes the differences would undoubtedly be very slight if detectable at all.

In explaining the more rapid digestion of the proteins of irradiated milk than of non-irradiated milk by pepsin and trypsin during the early stages of digestion, use may be made of the work of Wallen-Lawrence and Koch² who have demonstrated that boiled milk digests more rapidly than unboiled milk due to the destruction of anti-enzymes by boiling. It is possible that irradiation destroys or partially destroys the anti-enzymes in milk thus making irradiated milk more easily digested than milk not so treated.

SUMMARY AND CONCLUSION

A biochemical study has been made of milk and butter from milk treated with a heavy dose of ultraviolet light. Controls of non-irradiated milk and butter were also run. There is very little change in the composition of milk due to irradiation. Butter from irradiated milk has a shorter induction period for oxidation than that from non-irradiated milk. The variation in fat constants is slight. Digestion studies in vitro with pepsin and trypsin indicate a slight speeding up of digestion during the early stages of the process. This may be due to a destruction of anti-enzymes by ultraviolet irradiation. Normal irradiation by the method described would likely produce no detectable change in the composition or digestibility of milk.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Dr. Arthur G. Davis, of Erie, Pennsylvania, in overseeing the preparation of the samples for analysis. They also wish to thank Mr. George Taylor, of Erie, Pennsylvania, owner of the White Swan Farms, for the use of his facilities.

² Wallen-Lawrence, Z., and Koch, F. C. The relative digestibility of unsweetened evaporated milk, boiled milk and raw milk by trypsin in vitro. *Amer. Jour. Dis. Child.* 39: 18-33. 1930.

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A Colorimetric Method for the Determination of Tartaric Acid

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IN ATTEMPTING to develop a colorimetric method for the determination of tartaric acid or tartrates, various color reactions of tartaric acid were investigated. The only one which showed promise of being applicable is that first described by Fenton (2, 3). In this test a violet color is produced when a tartrate is treated with ferrous sulfate, hydrogen peroxide, and sodium hydroxide. This color reaction appears to be specific for tartaric acid. Fenton found that citric, succinic, malic, and oxalic acids and sugar do not give the test, and these observations have been confirmed by the authors.

In the following report a method is described whereby Fenton's color reaction for tartaric acid is made the basis of a quantitative colorimetric method for its determination. It has been found to give good results on solutions of pure tartaric acid, on tartrate baking powders, and on tartrate baking powders in the presence of aluminum.

PREPARATION OF REAGENTS

ONE PER CENT FERROUS SULFATE. Dissolve 1 gram of ferrous sulfate in 80 cc. of water, heating gently and stirring to aid solution. Cool, transfer to a 100-cc. volumetric flask, and make up to volume.

HYDROGEN PEROXIDE. Use a good grade of commercial 3 per cent hydrogen peroxide.

NORMAL SODIUM HYDROXIDE. Prepare an exactly normal solution of sodium hydroxide.

TARTARIC ACID STANDARD SOLUTION. Transfer 16 grams of dry *d*-tartaric acid to a 100-cc. volumetric flask, dissolve in water, and make up to volume.

WORKING TARTARIC ACID STANDARD SOLUTION. Transfer 5 cc. of the tartaric acid standard solution to a 100-cc. volumetric flask, add 10.66 cc. of normal sodium hydroxide solution and make up to volume. This solution contains 0.80 gram of tartaric acid per 100 cc. and has a pH of 6.2.

(1)

ANALYTICAL PROCEDURE FOR THE ANALYSIS OF A TARTRATE BAKING POWDER

Transfer to a small beaker a 2-gram sample of baking powder. Add water, drop by drop, until carbon dioxide ceases to be evolved. Next add 45 cc. of water and stir thoroughly to dissolve the tartrates present. To remove the starch, filter into a 100-cc. volumetric flask and wash the residue three times with 15 cc. of water at each washing. Make up to volume with water. This solution should have a pH of approximately 6.2. If the pH varies from 6.2 by more than ± 0.5 , another sample should be prepared and the pH adjusted before making up to volume. The pH of the solution may be determined colorimetrically, using chlorophenol red as an indicator. As a rule tartrate baking powders require no adjustment.

Transfer 10 cc. of the above solution to a 25-cc. volumetric flask. Add 0.2 cc. of 1 per cent ferrous sulfate solution and 0.2 cc. of hydrogen peroxide and mix thoroughly. Upon the addition of hydrogen peroxide the solution will turn yellow. Allow the solution to stand until it becomes brownish in color and then place it in an ice bath until the brown color disappears and the color becomes definitely lavender. Add immediately 5 cc. of normal sodium hydroxide solution. Stopper the flask, mix by inversion twice, and place the flask in the ice bath for ten minutes. At the end of this time remove the flask from the ice bath, mix by inversion twice, and compare in a colorimeter with a standard prepared simultaneously. For the standard, 10 cc. of the working tartaric acid standard solution containing 0.08 gram of tartaric acid are used. The results may be calculated from the following formula:

$$\frac{\text{Reading of standard} \times 0.08 \times 10 \times 100}{\text{Reading of unknown} \times \text{wt. of sample}} = \% \text{ tartaric acid}$$

In Table I are given the analyses of two samples of tartrate baking powder for total tartaric acid by the colorimetric method and by the official method (1) of the Association of Official Agricultural Chemists. It will be noted that the colorimetric method in both cases gives slightly higher results than the A. O. A. C. method. The lower results in the A. O. A. C. method may be due to incomplete precipitation of potassium acid tartrate or its slight solubility in the alcohol which is used in washing the precipitate.

In order to check further the reliability of the colorimetric method, samples of baking powder were prepared containing known amounts of tartaric acid. In Table II results of analyses of these samples by the colorimetric method are given.

It was suggested to the authors that the colorimetric method would be especially valuable in the analysis of baking powders if it gave accurate results in the presence of aluminum. To test the method in the presence of aluminum, 5 grams of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ were mixed with 95 grams of

(2)

baking powder whose tartaric acid content was known. By calculation the mixture contained 38.24 per cent of tartaric acid. The results of five analyses by the colorimetric method gave values ranging from 38.09 to 38.46 per cent, with an average value of 38.31 per cent. It is evident that the presence of aluminum does not interfere with the colorimetric determination of tartaric acid in a tartrate baking powder.

TABLE I. ANALYSES OF TARTRATE BAKING POWDERS FOR TOTAL TARTARIC ACID

COLORIMETRIC METHOD %		A. O. A. C. METHOD %
	SAMPLE 1	
39.8		39.6
40.0		39.4
39.6		39.6
39.6		39.7
39.8		39.5
Av. 39.76		39.56
	SAMPLE 2	
34.8		34.3
34.6		34.2
34.5		34.2
34.8		34.3
34.6		34.2
Av. 34.66		34.24

TABLE II. ANALYSES OF BAKING POWDERS BY THE COLORIMETRIC METHOD

TARTARIC ACID PRESENT				
%	%	%	%	%
10.00	15.00	20.00	30.00	40.00
TARTARIC ACID FOUND				
9.95	14.92	20.00	30.00	39.80
10.00	14.92	19.90	30.15	40.00
9.95	15.00	20.00	29.85	40.00
10.00	15.00	20.20	30.00	40.21
Av. 9.98	14.96	20.03	30.00	40.00

As a matter of interest the application of the colorimetric method to the determination of other forms of tartaric acid was studied. Using *d*-tartaric acid as a standard, it was found that *l*-tartaric acid, *l*-ammonium tartrate, and *meso*-tartaric acid produce a color equivalent to that of the standard. With racemic acid the color intensity was approximately one-half that of the standard.

This reaction of racemic acid was surprising. It was thought that possibly there might be some union of the *d*- and *l*-forms in racemic acid which was causing an interference in the reaction, but molecular weight determinations by the freezing-point method indicates no such union. With regard to the purity of the racemic acid used (obtained from the Eastman Kodak Company) it may be said that it was optically inactive and that it required the theoretical amount of sodium hydroxide for neutralization. The melting point was 202° C., whereas the accepted value is 205–206° C. A mechanical mixture of equal parts of *d*- and *l*-tartaric acids did not react like racemic acid but gave the proper color intensity. Two different samples of racemic acid were analyzed with identical results. No satisfactory explanation can be made for this behavior of racemic acid. Racemic

(3)

acid crystallizes with one molecule of water of crystallization, while *d*- and *l*-tartaric acids crystallize in the anhydrous form. This might suggest some difference between the chemical properties of racemic acid and the *d*- and *l*-forms of tartaric acid. *Meso*-tartaric acid also crystallizes with one molecule of water, but it gives the same color as the *d*- and *l*-forms. Hence it appears that the water of crystallization is not a factor in color production.

With regard to the use of this method, the authors feel that certain points should be emphasized. The pH of the standard and of the unknown should be approximately the same at about 6.2. The sample taken should be of such a size that the color of the standard and of the unknown is approximately the same. The amount of ferrous sulfate used should be exactly 0.2 cc. The sodium hydroxide solution should be added as soon as the lavender color appears. The method is not applicable in the presence of calcium or phosphates.

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- (1) Assoc. Official Agr. Chem., Official and Tentative Methods, p. 307 (1925).
- (2) Fenton, H. J. H., *Chem. News*, 33, 190 (1876).
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(4)

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THE CARBON METABOLISM OF FUSARIUM OXYSPORUM ON GLUCOSE

BY

ARTHUR K. ANDERSON, EDWARD L. EVERITT
AND PHILIP D. ADAMS

(Contribution from Pennsylvania Agricultural Experiment Station)

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THE CARBON METABOLISM OF FUSARIUM OXYSPORUM ON GLUCOSE¹

By ARTHUR K. ANDERSON, *Physiological Chemist*; EDWARD L. EVERITT and
PHILIP D. ADAMS, *Pennsylvania Agricultural Experimental Station*

INTRODUCTION

A very important phase of research in plant pathology is that which deals with the physiological relationships existing between the causative organism and the host. Any information on the biochemistry of the causative organism should prove of value in studying the physiology of a plant disease. It is very probable that some metabolic product of the organism is the important factor producing the symptoms of the disease in the plant.

REVIEW OF LITERATURE

In the past considerable work has been done on the biochemistry of *Fusarium lini* Bolley, the organism causing flax wilt. In 1920 Tochinai (9, 10, 11)² reported a rather complete study of the food requirements of this organism. In 1924 Anderson (1) repeated much of the work of Tochinai and also studied the products of metabolism of this organism on glucose and xylose media. He found that the organism produced a rather typical alcoholic fermentation on glucose. About 90 per cent of the original carbon could be accounted for in the products of metabolism studied. In the case of xylose about 80 per cent of the original carbon could be accounted for in the ethyl alcohol, carbon dioxide, and mycelium produced. He suggested the possibility that the alcohol caused the wilt.

In 1926 Letcher and Willaman (6) attempted to correlate the pathogenicity of various strains of *Fusarium lini* with their ability to produce ethyl alcohol. They examined nine strains of the fungus and found that the two which were the least virulent on flax produced the least alcohol. It should be noted, however, that the strain which produced the most alcohol was not the most virulent.

In 1928 White and Willaman (14, 15) studied the fermentation of pentoses by *Fusarium lini* and accounted for nearly 100 per cent of the original carbon in the form of carbon dioxide, ethyl alcohol, and mycelium. They also reported the growth of the organism on the postulated intermediate products formed by yeast in the pyruvic acid theory of fermentation. They believed that *F. lini* ferments glucose by the same mechanism as that of yeast.

Reynolds (8) in 1926 reported on the utilization of various carbohydrates and nitrogen compounds by *Fusarium lini*.

In 1931 Birkinshaw, Charles, Raistrick, and Stoye (2) reported on the carbon metabolism of various species of *Fusarium*, including *F. oxysporum*, grown on a glucose medium. They found some variation among the various species but all of them produce ethyl alcohol.

¹ Received for publication July 22, 1932; issued April, 1933.

² Reference is made by number (italic) to Literature Cited, p. 481.

PURPOSE OF THE INVESTIGATION

The purpose of the present work has been to open the way for an investigation of another wilt-producing fungus, *Fusarium oxysporum* Schlecht., using the same type of medium and employing essentially the same methods of determining the products of metabolism as were employed by Anderson (1) on *F. lini*. This has made it possible to compare the metabolism of these two organisms under practically the same conditions. The principal part of the study has been to determine the quantitative distribution of the chief products of metabolism (ethyl alcohol, carbon dioxide, and mycelium) of *F. oxysporum* on glucose. A further study has been made to determine whether or not ethyl alcohol, in concentrations equal to that produced by *F. oxysporum* in artificial cultures, is toxic to potato cuttings.

METHODS

SOURCE OF FUSARIUM OXYSPORUM CULTURE

The culture of the organism used in this work was obtained from the mycological herbarium of the University of Minnesota through the courtesy of Dr. Louise Dosdall. This was isolated in 1919 by Dr. G. R. Bisby (3) in connection with his studies on the *Fusarium* diseases of potatoes and truck crops.

CULTURE MEDIA

The mineral medium used in this work was the same as that used by Tochinal (9) and Anderson (1) in their work on *Fusarium lini*. It has the following composition:

Ammonium nitrate.....	1.00 g ³
Magnesium sulphate.....	.25 g
Monopotassium phosphate.....	.50 g
Water to make.....	1,000 c c

To this stock solution sufficient glucose was added to make an approximately 2 per cent solution of glucose. The exact quantity of glucose was determined after sterilization by the Folin-Wu (5) method.

HYDROGEN-ION CONCENTRATION

Hydrogen-ion concentration determinations were made by the electrometric method using the quinhydrone electrode.

CULTURE FLASKS AND TEMPERATURE OF INCUBATION

The culture flasks used were 500 c c Erlenmeyer flasks fitted with 2-hole rubber stoppers. Glass tubes were placed through the holes of the stoppers and bent at right angles just above them. One of the tubes extended just below the stopper and the other reached to the bottom of the flask. The outer end of each tube was plugged with cotton and fitted with a rubber tube that could be closed by a pinchcock. Into each flask 300 c c of the medium were placed and the long tube was drawn up so that the lower end was about an inch above the surface of the liquid. The flasks were then sterilized at 15 pounds pressure for 20 minutes. When cooled each flask was inoculated by the addition of 5 c c of a spore and mycelium suspension of the

³ g is the abbreviation for gram or grams recently adopted by the Style Manual for U. S. Government printing.

organism in water. The stoppers were then adjusted and sealed with paraffin and the rubber connections closed with pinchcocks.

The flasks were kept at room temperature in a laboratory where the temperature varied only slightly from 25° C.

DETERMINATION OF CARBON DIOXIDE

Carbon dioxide was determined at frequent intervals throughout the experiment in order to prevent loss of this product of metabolism. The method used was to aerate each culture flask into barium hydroxide solution contained in a Truog (12) tower fitted into a 500 c c suction flask through a rubber stopper. The air used for aeration was freed from carbon dioxide by passing it through soda lime. In order to prevent loss of alcohol, a Truog tower containing concentrated sulphuric acid was placed ahead of the barium hydroxide tower. A separate sulphuric acid tower was prepared for each culture flask. These were used every time a culture flask was aerated for carbon dioxide and finally the alcohol was determined, as will be described later. After aeration the excess of barium hydroxide was titrated with standard hydrochloric acid solution using phenolphthalein as an indicator. Before flasks were removed for final analysis the long tube was pushed below the surface of the liquid and aerated for 30 minutes in order to remove the last traces of carbon dioxide. Each time the flasks were aerated for carbon dioxide a blank determination was made. The titer of the carbon dioxide determinations was subtracted from this blank and the difference used in calculating the carbon dioxide in the sample.

DETERMINATION OF DRY MATTER AND CARBON IN THE MYCELIUM

When the experiment was started a sufficient number of culture flasks were prepared so that duplicate samples could be analyzed at intervals of a week or 10 days over a period of about two months. The weight of mycelium was determined by filtering through a weighed Gooch crucible and washing with water. The pad of mycelium was then dried to constant weight at 100° C. The filtrate was made up to a volume of 500 c c and aliquot portions were used for subsequent determinations.

The carbon in the mycelium was determined by the wet combustion method using the Knorr apparatus and the details of procedure as outlined by White and Holben (13). The entire mycelium mat, together with the asbestos, was introduced into the digestion flask.

DETERMINATION OF ALCOHOL

For the determination of ethyl alcohol the method of Dox and Lamb (4) was used with modifications. In this method the alcohol is oxidized to acetic acid which is distilled and titrated with standard alkali. After filtering and washing the mycelium and making up the filtrate to a volume of 500 c c, a 300 c c aliquot of the filtrate was saturated with solid ammonium sulphate and the mixture aerated for 48 hours into a Truog tower containing concentrated sulphuric acid. The acid mixture, together with the glass beads, was then transferred to a 2-liter Claisen flask containing 22 g of potassium dichromate. After standing for 20 minutes the resulting acetic acid was distilled and titrated with standard alkali. In this distillation the flask was

heated until foaming occurred. Next 100 c c of carbon-dioxide-free water were added to the distilling flask, the distillation was repeated, and the distillate titrated. This last procedure was repeated until a constant titer was obtained. This represents the amount of sulphuric acid unavoidably distilled at each distillation, and the constant titer times the number of distillations represents a blank which must be subtracted from the sum of all the titrations.

Since in the determination of carbon dioxide a sulphuric acid tower was always placed ahead of the barium hydroxide tower to collect any alcohol given off, another alcohol determination was made on the contents of this tower. The quantity of alcohol found was added to that found in the filtrate to give the total alcohol produced in a given flask.

DETERMINATION OF GLUCOSE

Glucose was determined by the method of Folin and Wu (5). An aliquot of the filtrate from the mycelium determination was diluted so that 1 c c contained about 0.1 mg of glucose. Under these conditions the color of the unknown matches closely that of the Folin and Wu dilute glucose standard.

PRESENTATION OF DATA

Sixteen flasks with 300 c c of medium containing a known quantity of glucose as the only source of carbon were inoculated with a spore and mycelium suspension of *Fusarium oxysporum*. These were stoppered and the aeration tubes were closed by means of rubber tubes and pinchcocks in order to prevent loss of carbon dioxide. Carbon dioxide was determined at frequent intervals. During the active growth of the organism it was determined daily. At intervals of a week or 10 days two flasks were removed and analyzed for residual carbon dioxide, carbon in the mycelium, glucose, and ethyl alcohol. The original culture medium was analyzed for glucose and the pH determined. The last two flasks were used for pH determinations.

TABLE 1.—Distribution of metabolic products of *Fusarium oxysporum* on a glucose medium at various stages of growth

Age of culture (days)	Mycelium				Carbon dioxide		Ethyl alcohol		Glucose		Total recovery of carbon as related to initial carbon ^a	pH
	Weight	Weight of carbon	Carbon	Carbon as related to initial carbon ^a	Weight of carbon	Carbon as related to initial carbon ^a	Carbon in 300 c c	Carbon as related to initial carbon ^a	Carbon in 300 c c	Carbon as related to initial carbon ^a		
	Gram	Gram	Per cent	Per cent	Gram	Per cent	Gram	Per cent	Gram	Per cent	Per cent	
0	0.1399	0.0651	46.53	3.15	0.1998	9.68	0.3501	16.97	2.0633	100	108.17	4.15
12	.2466	.1129	45.78	5.47	.5150	24.96	1.0153	49.21	1.6170	78.37	80.33	
20	.2432	.1095	45.02	5.31	.5389	26.12	1.0172	49.30	.0141	.68	80.72	
27	.2484	.1140	45.89	5.52	.5457	26.45	1.0379	50.30			82.23	
36	.2440	.1118	45.82	5.42	.5779	28.01	1.0707	51.89			85.32	
46	.2399	.1204	50.18	5.83	.6376	30.90	1.0030	48.61			85.34	
59	.1929	.1027	53.24	4.98	.7051	34.17	.9878	47.88			87.03	6.55

^a Expressed as percentage of the carbon in the glucose originally present.

^b 1 determination.

In all, four experiments were run. In Table 1 and Figure 1 the data are given for one representative experiment. It should be noted that results are expressed in terms of carbon and also in terms of percentage of the carbon of the glucose originally present in each flask.

Referring to Table 1 and Figure 1, the following facts are brought out concerning the progressive changes in the products of metabolism.

THE MYCELIUM AND ITS CARBON CONTENT

The weight of mycelium increases rapidly, reaches a maximum at 36 days, and finally decreases. The maximum is reached at the time that all the glucose has been consumed. Apparently stored material

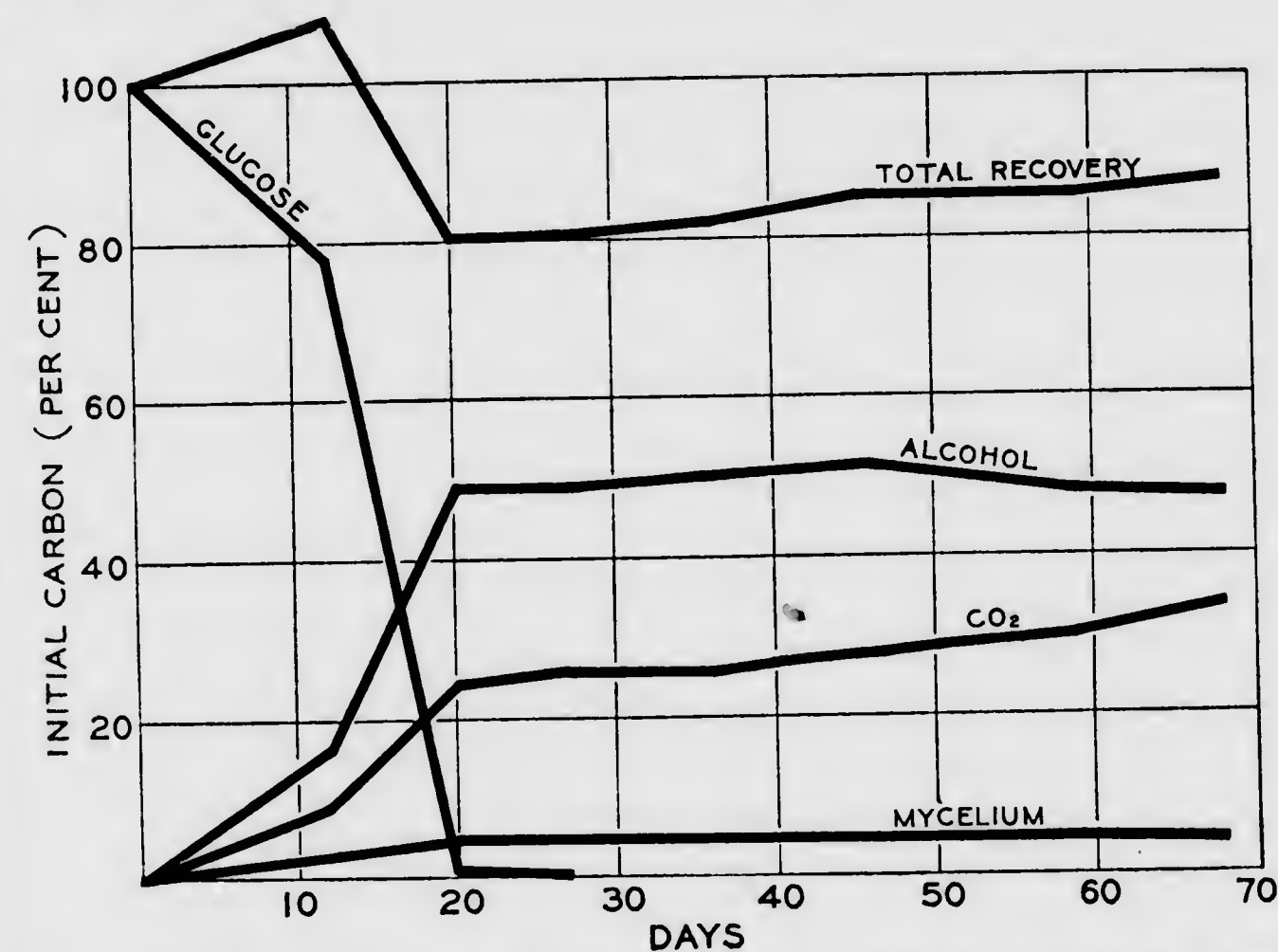


FIGURE 1.—Distribution of metabolic products of *Fusarium oxysporum* when grown on glucose

in the mycelium is consumed after other sources of food have been utilized.

The weight of dry mycelium produced approximates that found by Anderson (1) for *Fusarium lini*, although he used only one-third as much medium. He found as high as 16.75 per cent of the original carbon in the mycelium, while with *F. oxysporum* 5.83 per cent is the maximum. *F. lini* shows a very definite utilization of ethyl alcohol for mycelium production. *F. oxysporum* is apparently unable to utilize ethyl alcohol for mycelium building purposes.

The percentage of carbon in the mycelium remains very constant at about 46 per cent for 46 days when it increases to a final value of over 53 per cent. This increase in carbon content of mycelium with age was also noted by Anderson (1) in his work on *Fusarium lini*. Since carbohydrates contain about 40 per cent of carbon, proteins from 51 to 55 per cent, and a typical fat such as tristearin 76.87 per cent, Anderson believes that the increase of carbon in the mycelium indicates

a change of the carbohydrate in young mycelium into fat as the mycelium ages. Since the percentage of nitrogen in the mycelium decreased with age, he felt there was not a change of carbohydrate to protein. Although nitrogen was not determined on the mycelium in this work on *F. oxysporum*, it is a reasonable assumption that the increase in carbon content is due to a conversion of carbohydrate into fat.

The percentage of original carbon found in the mycelium is well over 5 during active growth. In the last analysis this drops to 4.98 per cent, which indicates a utilization of carbon in the mycelium for food purposes as other sources of food are consumed.

CARBON DIOXIDE

Carbon dioxide is produced throughout the experiment. During the early part of the experiment carbon dioxide was produced very rapidly, necessitating its determination daily. When the glucose is all consumed, the rate of carbon dioxide production decreases so that it is necessary to determine it only at infrequent intervals. The percentage of original carbon in the form of carbon dioxide increases throughout the experiment, reaching a maximum of 34.17 per cent in the final analysis.

ETHYL ALCOHOL

Ethyl alcohol is produced rapidly by *Fusarium oxysporum*. In 20 days the percentage of original carbon in the form of ethyl alcohol is 49.21. This increases gradually to 51.89 per cent at the end of 46 days. From then on the quantity of alcohol decreases, falling to 47.88 per cent of the original carbon at the end of the experiment. Anderson (1) in his work on *F. lini* found that this organism converted 52.75 per cent of the original carbon into alcohol when the amount of alcohol was at a maximum. At the end of the experiment the original carbon in the form of ethyl alcohol was only 29.51 per cent. *F. lini* very definitely utilizes ethyl alcohol as a food material. There is only slight utilization of ethyl alcohol by *F. oxysporum*.

GLUCOSE

The glucose of the medium is consumed rapidly. In 20 days there is only a trace left. Since the total recovery of carbon at the end of the 12-day period is 108.17 per cent, it is felt that the error is in the determination of glucose. It is very possible that in the early stages of growth the organism converts glucose into intermediate products which have the power of reducing copper solutions. If this is true, the value for glucose at the 12-day period is high.

TOTAL RECOVERY OF CARBON

In the column headed "total recovery" in Table 1 are recorded the sum of the values obtained for the various products of metabolism. It will be noted that at the end of the 12-day period the recovery is 108.17 per cent. As pointed out above this is likely due to the production of intermediate products which reduce copper solutions, rendering the glucose determination high. At the end of the 20-day period the total recovery has fallen to 80.33 per cent. From then on the total recovery gradually increases until at the end of the experiment it is 87.03 per cent. The fact that all of the carbon is not

accounted for indicates that there are other products of metabolism than those studied, although some of the loss may be due to incomplete recovery of some of the products studied.

HYDROGEN-ION CONCENTRATION

Determinations of pH were made only at the beginning and at the end of the experiment. The initial pH was 4.15 and the final pH was 6.55. Anderson (1) in his work on *Fusarium lini* found that this organism changed the pH of the medium toward the optimum for growth. It appears that the same occurs with *F. oxysporum*.

RATIO OF PRODUCTS OF METABOLISM TO ONE ANOTHER AND TO GLUCOSE CONSUMED

The relationships existing between the products of metabolism and the compounds consumed by a fungus have been expressed by means of various ratios and percentages. Such terms as respiration coefficient, economic coefficient, respiration equivalent, plastic equivalent, etc., have been introduced into the literature. There has been such a lack of uniformity with regard to the usage of these terms that in Table 2 a formula is placed at the head of each column to make perfectly clear the sense in which these terms are used in this discussion. They are used in the same sense as used by Peterson, Fred, and Schmidt (7).

The respiration coefficient represents the grams of carbon dioxide produced per gram of dry mycelium. It will be noted from Table 2 that this coefficient gradually increases with the age of the culture. Finally there is a rapid increase due to the consumption of the mycelium itself as a food material. The values are very much higher than those found by Anderson (1) for *Fusarium lini*.

TABLE 2.—Quantitative relationships existing between the various metabolic products of *Fusarium oxysporum* when grown on a glucose medium

Age of culture (days)	Respiration coefficient, weight of CO ₂ weight of mycelium	Economic coefficient, weight of sugar consumed weight of mycelium	Respiration equivalent, carbon of CO ₂ ×100 carbon consumed	Plastic equivalent, carbon of mycelium×100 carbon of glucose consumed	Alcohol equivalent, carbon of alcohol×100 carbon of glucose consumed	Carbon of alcohol carbon of CO ₂
12	5.24	7.98	44.76	14.60	78.45	1.75
20	7.66	20.78	25.03	5.51	52.09	1.97
27	8.13	25.32	26.12	5.31	49.30	1.88
36	8.06	24.79	26.45	5.52	50.30	1.90
46	8.68	25.24	28.01	5.41	51.90	1.85
59	9.75	25.66	30.90	5.83	48.61	1.57
68	13.40	31.92	34.17	4.98	47.90	1.40

The economic coefficient represents the grams of glucose necessary to produce 1 g of dry mycelium. This coefficient increases with the age of the culture, reaching a maximum of 31.92 in 68 days. Anderson (1) found a maximum value of 13.1 with *Fusarium lini*.

The respiration equivalent is the percentage of carbon consumed that is transformed into carbon dioxide. With the exception of the 12-day period, where the value is high due to a false sugar value, this

equivalent increases with age. Anderson (1) found slightly higher values for *Fusarium lini*.

The plastic equivalent represents the percentage of carbon source used in mycelium synthesis. Here again is found a high value at the 12-day period due to a false sugar value. The other values are approximately constant but lower than those found by Anderson (1) for *Fusarium lini*.

The alcohol equivalent is a new term that the authors have introduced which expresses the percentage of carbon of the glucose consumed that is converted into alcohol. At the end of the 12-day period the value is 78.45 which is undoubtedly too high due to a false glucose value as mentioned above. For the remaining periods the value drops to around 50. In the last two periods the value drops due to consumption of the alcohol by the fungus.

In the last column the ratio of carbon in alcohol to carbon in carbon dioxide is given. In a typical alcoholic fermentation glucose is decomposed according to the following equation:



In this equation the ratio of carbon in alcohol to carbon in CO_2 is 2:1. It will be seen from Table 2 that at the end of the 20-day period the value is 1.97:1, which is very close to the theoretical for a typical alcoholic fermentation. From this point on the ratio decreases until at the end of the experiment it is 1.40:1. Here again utilization of the alcohol by the fungus is indicated.

In the early part of this study an error was made in making up the nutrient mineral solution. In place of 1 g of NH_4NO_3 per liter, 100 g per liter were used. It is felt worthy of mention that *Fusarium oxysporum* grew on this medium and produced a typical alcoholic fermentation. When the distribution of the products of metabolism were charted as in Figure 1, in general, the curves were the same. The main difference between the two figures was that the rate of production of the various products of metabolism on the strong mineral solution was about one-third as fast as on the weak mineral solution.

THE EFFECT OF ALCOHOL ON POTATO CUTTINGS

Anderson (1) has suggested that the toxicity of *Fusarium lini* to flax may be due to the production of ethyl alcohol by the organism in the plant tissue. When it was ascertained that *F. oxysporum* produces ethyl alcohol on glucose it became of interest to study the effect of solutions of ethyl alcohol at various concentrations on potato cuttings.

Solutions of alcohol were made, varying in concentration from 0.5 to 5 per cent in increments of 0.5 per cent. Potato cuttings were placed in these solutions in beakers. Several cuttings were placed in tap water as a control. In no case was there any appreciable change within 24 hours, other than a slight curling of the leaves. After 48 hours a mottled yellow began to appear on the leaves of the cuttings in alcohol. This did not occur in the controls. The stems remained firm. When the cuttings in alcohol were shown to a pathologist familiar with potato diseases, he believed they were affected with an extreme case of leaf mosaic. There was no wilting of the stems even

at the end of five days. A very striking odor resembling that of ripe cantaloupes was given off by the cuttings in alcohol.

Since in all of the above work the stems remained firm, it was decided to determine the concentration of alcohol necessary to produce definite and rapid wilting. Cuttings were placed in solutions containing from 10 to 55 per cent of alcohol in increments of 5 per cent. Within two hours there was a general wilting in all cases, the wilting increasing in severity with the higher concentrations. There was no appreciable change in color of the leaves as was noted with the more dilute solutions of alcohol. The cantaloupe odor previously noted was very strong in all cases. Judging from the esterlike odor, one is led to the hypothesis that the potato plant is able partly to protect itself against ethyl alcohol by converting it to an ester.

The findings in solutions of alcohol up to 5 per cent are in agreement with the work of White (16) who observed no ill effects on tomato cuttings in solutions of ethyl alcohol up to 4 per cent.

SUMMARY AND CONCLUSIONS

It is evident from the data presented that the main products of metabolism of *Fusarium oxysporum* on glucose are carbon dioxide and ethyl alcohol. The proportion of these two compounds formed indicates that the organism causes a rather typical alcoholic fermentation. In this respect *F. oxysporum* is similar to *F. lini*.

Fusarium lini very definitely uses ethyl alcohol as a source of food supply while *F. oxysporum* makes only slight use of this product.

Work with potato cuttings in ethyl alcohol solutions would indicate that this product is not responsible for the wilting of potato plants. It is likely that small quantities of alcohol are rendered nontoxic by conversion into an ester by the potato plant. Potato cuttings in a 10 per cent alcohol solution showed very definite symptoms of wilting. It is quite possible that in this case the concentration of alcohol in the tissue was much less than 10 per cent. Although there is no experimental evidence to support the theory that alcohol is responsible for the wilting, it is reasonable to suppose that alcohol may at least be a factor in the production of the symptoms of wilt in potato plants infected with *Fusarium oxysporum*.

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A CHEMICAL STUDY OF RANCIDITY III. SOME RECENT DEVELOPMENTS IN THE STUDY OF OXIDATIVE RANCIDITY OF SPECIAL INTEREST TO THE CEREAL INDUSTRY¹

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(Read at the Convention, June, 1933)

An increasing interest is being evidenced by various branches of the food industry in the spoilage of foods through rancidity development. The first industries to be concerned were the manufacturers of butter and edible shortenings due to the necessity of producing a product that could be stored and marketed without appreciable deterioration. The bakers of certain goods, particularly crackers, also became interested because of the possibilities of rancidity development in their product. The advent, during the past few years, of prepared and packaged biscuit flours and doughs containing large amounts of shortening agents has also necessitated a serious consideration of rancidity problems. From a nutritional standpoint it has been demonstrated that rancid fats exert a destructive effect on certain of the vitamins. These facts emphasize the widespread occurrence of rancidity problems in the food industry and the reason for considering some advances made in this study during the past few years.

Terminology

One encouraging advance of the past few years in regard to a clearer understanding of rancidity problems has been the more careful use of the term rancidity. In the past, this term has been used rather loosely to include any or all of the three types of fat spoilage, which have been classified by Triebold (1931) as oxidative, hydrolytic, and ketonic rancidity.

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At present, a group of investigators, considering only one particular fat, are using the term rancidity in a very restricted sense to include only that spoilage where butyric acid is liberated. The majority of investigators, however, dealing with the numerous fats and oils of industry are using the term to include all the types of rancidity, but with enough description to clearly designate the type involved. This more careful use of the term has eliminated much of the past confusion regarding the causes, effects, and methods of preventing rancidity. Since oxidative rancidity is the type usually encountered in the cereal industry, it is the only type considered in this paper.

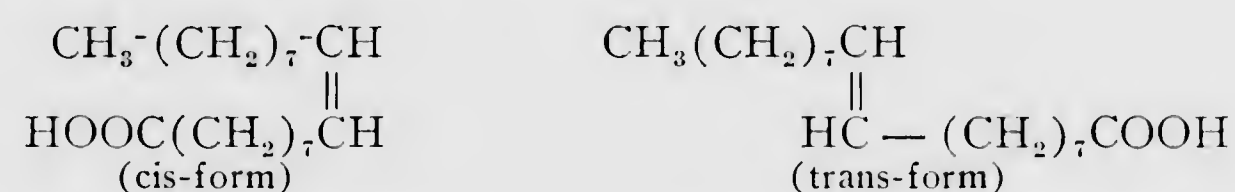
Susceptibility of Fats to Oxidative Rancidity

Considerable variations in susceptibility to oxidative rancidity are experienced between individual samples of the same type of fat as well as between different types. Various reasons have been suggested to account for this difference in behavior of the fats, and, it is likely, that all the reasons advanced play a part in the final explanation of this phenomenon.

Differences in Susceptibility to Oxidative Rancidity between Types of Fats

Without a doubt, the difference in susceptibility to oxidative rancidity between the various types of fats is influenced, in a large measure, by the character of their fatty acids combined as glycerides. It is obvious, that if oxidative deterioration involves particularly the unsaturated acids, as all theories postulate, then those fats containing the lesser amount of unsaturated glycerides, and, more especially, the lesser amount of highly unsaturated ones such as linolic and linolenic acid glycerides, will have the least susceptibility to oxidative rancidity. The excellent keeping qualities of cocoanut oil, consisting principally of saturated acid glycerides, appears to confirm this idea. So also does the improved keeping qualities of cottonseed oil after selective hydrogenation of the highly unsaturated glycerides.

Another interesting example of the influence of structure on the ease of oxidation is illustrated in the work of Täufel and Spiegelberg (1930). They have shown that in the case of the cis-trans isomers, oleic and elaidic acids,



that the oleic acid reacts much more readily with oxygen than its isomer, elaidic acid. The difference in reactivity between these two acids has

suggested to us another reason for the greater resistance to oxidation of hydrogenated shortenings. It had been pointed out by Hilditch (1927) that

"Since the ethylenic linkages in a highly unsaturated fatty acid occur at different points in the chain of carbon atoms, it is easy to understand why the oleic acids produced by selective hydrogenation are not all the same and identical with the ordinary oleic acid $\Delta^9:10$ -octadecanoic acid—the double bond left unsaturated may well be one of the others."

In our opinion, this effect of hydrogenation results in the formation of isomeric oleic acid glycerides differing from the naturally occurring oleic acid glycerides not only in physical properties but also in reactivity towards oxygen.

Differences in Susceptibility to Oxidative Rancidity between Individual Fats of the Same Type

Individual fat samples within a type exhibit considerable variations with respect to susceptibility to oxidative rancidity. This can be accounted for on the basis that fats vary in their content of pro-oxygenic catalysts due to their past treatment and to contamination with foreign materials. Such conditions of past treatment which favor the formation of peroxide or moloxide compounds (high oxidizing potential) will increase susceptibility to autoxidation according to the amount of these peroxide compounds formed. Contamination of the fat with certain metals tremendously influences the susceptibility to autoxidation. Davies (1932) lists the following metals in the order of their effect as pro-oxygenic catalysts: (1) Vanadium, (2) copper, and (3) iron, nickel, and manganese.

Triebold (1929) suggested that differences in susceptibility of individual fats to autoxidation, as measured by the length of induction period, might be accounted for by varying amounts of anti-oxygenic substances contained in the fats. A similar idea has been suggested recently by Hilditch and Sleightholme (1932), who have demonstrated by experiments on four oil samples, that the original oils were more resistant to autoxidation than either the saponified oil, re-esterified fatty acids of the oils, or glycerides prepared synthetically from the distilled acids of the oils. It is very likely that the susceptibility to autoxidation of many of the fats and oils, particularly those which do not undergo a drastic refining process, is influenced to a considerable degree by the presence of anti-oxygenic substances.

Methods of Determining the Susceptibility of Fats to Oxidative Rancidity

Several investigators (Holm and Greenbank, 1923; Greenbank and Holm, 1925, 1930; Hilditch and Sleightholme, 1932; Mattill, 1931;

Triebold and Bailey, 1932) have used controlled oxidation studies to determine the effect of various factors in the oxidative deterioration of fats. Although all these methods are the same in principle, they vary considerably in detail. For example, variations in the temperature of autoxidation may range from room temperature to 95° C., stirring is employed in some methods and not in others, while one method uses light as a catalyst instead of heat.

We have made a comparison of the results obtained by controlled autoxidation of lards at 95°, 90°, 70° C. without stirring, and 95° C. with stirring, using the Holm and Greenbank (1923) gas-tight stirrer, and have found, in general, that all the methods rank the samples in approximately the same relative order with respect to keeping ability. Stirring does not appear to influence the results as evidently there is no appreciable surface film formation through polymerization, although this would likely be a factor in the case of the drying oils. Since this study was made, we have adopted, when stirring is necessary, the mercury seal stirrer as used by Briggs (1931) thereby eliminating the inherent difficulties of the Holm and Greenbank stirrer, namely, the danger of contamination with metallic catalysts, and of not obtaining an absolutely gas-tight seal in the bearing. There is a possibility that if the fat contains metal catalysts, temperatures over 75° C. may lead to erroneous results. Ellis (1932) has demonstrated that using cobaltous oleate as a catalyst, he could effect a considerable autoxidation of even the saturated fatty acids such as stearic acid at temperatures over 75° C. However, in the absence of catalyst, he got no oxidation of the saturated acids at the higher temperatures. We have found in the case of pure myristic acid, that even after 25 hours exposure to oxygen at 90° C. it had not begun to autoxidize. These results would tend to indicate that ordinarily the choice of a controlled oxidation method would limit itself to the ease and speed of obtaining results.

Method of Determining the Susceptibility of Crackers to Oxidative Rancidity

It is now generally accepted that the length of induction period of a fat, as determined by any of the controlled oxidation methods, is a good index of the susceptibility of that fat to oxidative rancidity. It was thought likely that a comparable relationship would hold between the length of induction period of a fat and the susceptibility to oxidative rancidity of crackers in which the fat was used as a shortening agent. While, in general, it was found by Triebold and Bailey (1932) that such a relationship seemed to exist, several outstanding exceptions were noted where the keeping quality of a fat baked into crackers was greatly enhanced over that indicated by the length of induction period of the

fat itself. However, the length of induction period determined on the crackers themselves was found to be a much better criterion for judging the susceptibility of crackers to oxidative rancidity.

Since the results of Triebold and Bailey (1932) were obtained on commercial crackers produced under very dissimilar conditions it was thought advisable to conduct a similar type of study on experimental crackers baked under standard conditions and thereby eliminating all variables except the shortening agent. Ten samples of lard shortenings were secured and baked into experimental crackers, attempting as much as possible to duplicate the formulas and procedures used in the baking of commercial crackers.

All the lards were autoxidized at 95° C. without stirring in an apparatus similar to that described by Triebold and Bailey (1932) to determine the lengths of their induction periods. Free acidities were determined according to the official method (A. O. A. C. Book of Methods, 1931, p. 326). The original method of Blunt and Feeney (1915) was used to obtain the smoking temperatures since the method as modified by McCoy (1931) did not yield better results. The peroxide contents as measured by the active oxygen values were determined by the method of Lea (1931) and reported as the cubic centimeters of N/500 sodium thiosulphate solution required to react with the iodine liberated from potassium iodide by 1 gm. of fat. Schibsted's (1932) method was used to ascertain the amount of aldehydes present in the fat and calculated according to his formula as the fat-aldehyde value. To obtain a measure of the keeping qualities of the lards, samples were stored at room temperature and at 40° C. and the number of days determined until the samples possessed the characteristics of oxidative rancidity, i.e., gave a definitely positive Kreis test and the characteristic rancid odor.

Keeping quality of the experimental crackers was ascertained by storage tests at 40° C. and noting the number of days required before they developed a rancid odor. Lengths of induction periods were also determined on the crackers by autoxidation at 95° C.

The results of this study are summarized in Table I, together with all the definite coefficients of rank of correlation obtained between the factors considered. The coefficient of rank of correlation (Jackson, 1924) was preferred to the straight coefficient of correlation since it was not desired in this case to correlate absolute values but rather to determine ability to rank one factor in respect to another. It is appreciated that the number of samples used in this study was so small that any conclusions drawn are simply indicative of what may be expected to occur.

Triebold and Bailey (1932) found a close relationship between the length of induction period of commercial crackers and their keeping

qualities and this same relationship holds for the experimental crackers reported in Table I. The coefficient of rank of correlation found $+ .927 \pm .031$, exemplifies the ability with which it is possible to rank the crackers in order of their keeping qualities and emphasizes the fact that the determination of the length of induction period on crackers provides us with a rapid and yet accurate method for evaluating the keeping qualities of such baked goods.

TABLE I

A COMPARISON OF THE KEEPING QUALITY OF EXPERIMENTAL CRACKERS WITH VARIOUS CHARACTERISTICS OF THE LARD SHORTENINGS USED IN THEIR PRODUCTION

All definite coefficients of rank of correlation obtained between the various factors are indicated.

Crackers			Lards						
Sample number	Days sweet (Storage at 40° C.)	Induction period in hours	Days sweet (Storage at room temperature)	Days sweet (Storage at 40° C.)	Induction period in hours	Free acidity Mgs. of KOH per gram of fat	Smoking temperature	Active oxygen (cc. N/500 Na ₂ S ₂ O ₈ per gram fat)	Fat aldehyde value
10	31 +	5.25	0	0	0.66	0.886	197.5	9.20	2.70
7	31	4.50	0	0	0.50	0.836	185.0	2.80	1.80
9	30	3.87	50	30	4.25	0.875	200.0	1.50	0.25
8	29	3.45	11	—	1.00	0.842	191.0	2.30	0.50
1	25	4.00	63	32	1.25	0.673	187.0	4.55	2.00
3	24	3.25	78	30	5.16	0.954	198.0	1.60	0.25
6	20	3.20	—	15	3.75	0.757	224.0	1.52	0.28
2	20	3.08	66	25	2.75	1.038	187.0	2.50	0.44
4	20	2.50	26	15	2.00	0.870	197.0	2.30	1.30
5	13	2.70	24	10	1.08	2.637	163.0	1.65	0.10

COEFFICIENTS OF RANK OF CORRELATION

Between induction period of crackers and the days they remained sweet at 40° C.	$r = + .927 \pm .031$
Between lard remaining sweet at room temperature and at 40° C.	$r = + .851 \pm .069$
Between induction period of lards and the days sweet at room temperature.	$r = + .896 \pm .046$
Between induction period of lards and their active oxygen content.	$r = - .806 \pm .078$
Between fat aldehyde value of lards and their active oxygen content.	$r = + .867 \pm .055$

As would be expected, there was a fairly close relationship between the keeping qualities of the lard samples at room temperature and at 40° C. This substantiates the practice of using a comparatively simple incubation test at an elevated temperature as described by Schaal (1931) for the rapid determination of the keeping quality of fats. A close relationship was also evidenced between the keeping quality of the lards

at room temperature and the length of their induction periods which is in accordance with the generally accepted idea that the length of induction period of a fat is a good index of its keeping quality.

No definite correlation was found between the free acidities of the lards and their keeping qualities on storage or their lengths of induction periods. The acidity values for all the samples were very similar except one lard, which had a value three times as large as the rest, and which also evidenced poor keeping quality. These results are also in agreement with those reported by Triebold and Bailey (1932a) in which it was found that only exceptionally high or low acidities were correlated with the keeping qualities of a fat.

While no significant correlation was reported between the length of induction period of the lards and their smoking temperatures, a slight correlation was evident. A much closer relationship was exhibited, however, between the length of induction period and active oxygen values of the lards. It would be expected that the active oxygen (or peroxide) values, since they give an indication of the oxidizing potential already built up in the samples, would be closely related to the lengths of induction periods of the lards. Only two high active oxygen values were obtained among the samples (Nos. 10 and 1) and these both had short induction periods. A good correlation was also experienced between the active oxygen and fat-aldehyde values. This would seem to indicate that in the early stages of oxidation, at least, the formation of aldehydes proceeds at a rate related in some measure to that of the peroxides. In these early stages of oxidation it should be possible then to test equally well for oxidative rancidity by means of either peroxide or aldehyde tests. As the oxidation progresses it is questionable if this relationship continues since Schibsted (1932) reports several cases of strongly oxidized fats with very low fat-aldehyde values.

It is significant to note the absence of a definite correlation between the lengths of induction periods of the lards and the keeping qualities of the crackers produced from them. An examination of the data on individual samples recorded in Table I shows why such a relationship did not exist in this study. The two cracker samples exhibiting the best keeping qualities (Nos. 10 and 7) were produced from lards with the shortest induction periods. Two other cracker samples (Nos. 8 and 1), possessing very good keeping qualities, were also made from lards possessing relatively short induction periods. This study would seem to indicate that shortenings of poor keeping quality may be used to produce crackers of good keeping quality, and this emphasizes a very important fact—that one cannot justly evaluate a shortening for its use in cracker manufacture by simply making a study on the shortening itself.

Inferior Shortenings Produce Crackers of Superior Keeping Quality

Triebold and Bailey (1932) found in the case of commercial crackers that a relationship seemed to exist between the length of induction period of a fat and its keeping quality when baked into crackers. However, several outstanding examples were also reported by them where lards of exceptionally poor keeping quality were used to produce crackers possessing good keeping quality. A possible explanation offered for this behavior was that the wheat oil present in the cracker flour might influence the keeping quality of the crackers. Wheat oil could not be responsible, however, in the case of the experimental crackers since they were all made from the same flour. Thus some reasonable explanation was desired to account for the excellent keeping qualities possessed by some crackers made from shortenings of inferior keeping qualities.

A possible hypothesis was suggested to account for this behavior. It would seem logical to assume that in the case of certain shortenings which had received good treatment and had undergone no appreciable deterioration before being baked into crackers, that a relationship would likely exist between the lengths of their induction periods and the keeping quality of the crackers in which they were used. On the same basis, if such shortenings were old or mistreated so that they had undergone incipient oxidation, this should be reflected in the lengths of induction periods and keeping qualities of the shortenings. It should be conceivable, however, that such shortenings when baked into crackers might have these deteriorative effects destroyed in the baking process and the resulting crackers should then possess keeping qualities comparable to those expected from the fresh shortening. Thus it might be possible to account for the ability of an inferior shortening in the incipient stages of oxidative rancidity to be used in the production of crackers with superior keeping qualities.

A short study was outlined to test the validity of this hypothesis. Two lard samples, one an open kettle and the other a prime steam rendered, were autoxidized to definite oxygen absorptions. After determining the lengths of induction periods and active oxygen (or peroxide) values on the two control and six oxidized samples, they were baked into experimental crackers. Within a week after baking, the lengths of induction periods were determined on the crackers, and active oxygen values obtained on the fat extracted from the crackers with petroleum ether. A small fraction of each of the cracker samples was stored at room temperature but up to the present none of these samples have shown any signs of oxidative rancidity.

The results of this study, summarized in Table II, appear to confirm the validity of the hypothesis presented. It is clearly indicated that autoxidation of a lard to a limited degree increases the active oxygen

content considerably and decreases or entirely eliminates the induction period. Crackers produced from the lards with high active oxygen values show materially reduced active oxygen contents for their extracted fat. The lengths of induction periods of the cracker samples have been increased in all cases over the lengths of induction periods of the corresponding lard samples and bear no relationship to either the lengths of induction periods or active oxygen values of the lards.

TABLE II

A COMPARISON OF THE LENGTH OF INDUCTION PERIOD OF EXPERIMENTAL CRACKERS CONTAINING LARD SHORTENINGS OXIDIZED TO DIFFERENT DEGREES WITH THE LENGTH OF INDUCTION PERIOD OF THE LARDS AND, ALSO, THE ACTIVE OXYGEN VALUES OF THE LARDS AND THE EXTRACTED FAT FROM THE CRACKERS

Length of induction periods determined by the static method at 90° C.

	Lard shortenings		Crackers	
	Induction period in hours	Active oxygen content (cc. N/500 Na ₂ S ₂ O ₃ per gram of fat)	Induction period in hours	Active oxygen content (cc. N/500 Na ₂ S ₂ O ₃ per gram of fat)
Open kettle rendered lard Control	3.0	1.6	8.0	1.7
Open kettle rendered lard Oxidized to beginning of the induction period	0.75	3.6	8.0	5.8
Open kettle rendered lard Oxidized to 15 cc. O ₂ absorption per 100 gms. lard	0.0	14.7	8.0	5.0
Open kettle rendered lard Oxidized to 50 cc. O ₂ absorption per 100 gms. lard	0.0	39.5	8.0	3.2
Prime steam rendered lard Control	1.75	3.8	9.5	4.4
Prime steam rendered lard Oxidized to beginning of the induction period	0.0	13.9	9.5	3.2
Prime steam rendered lard Oxidized to 15 cc. O ₂ absorption per 100 gms. lard	0.0	21.9	5.0	3.7
Prime steam rendered lard Oxidized to 50 cc. O ₂ absorption per 100 gms. lard	0.0	41.5	5.0	9.3

The relatively long and similar induction periods of crackers produced from the open kettle rendered lards in the various stages of autoxidation are to be expected as due to the reduction of the active oxygen contents of the lards in the baking process, and thereby, the removal of pro-oxygenic catalysts formed in the preliminary autoxidation of the lard. This same relationship holds in the prime steam lard samples with the exception of one case (crackers produced from the

lard sample autoxidized to 15 cc. of oxygen per 100 gms. of lard), which had a shorter induction period than the other cracker samples of the same active oxygen content. No explanation can be offered to account for this result. The shorter induction period of the crackers produced from the most highly autoxidized prime steam lard is due, in all probability, to the higher active oxygen content of the crackers. A comparison of the active oxygen values for the two types of lards and their behavior when baked into crackers would seem to indicate that autoxidation, in the case of the prime steam lards proceeds to a greater degree per unit of oxygen absorbed than is true of the open kettle rendered lards.

While this preliminary study would seem to indicate that it may be possible for a baker to use a lard shortening which is in the incipient stages of oxidative rancidity and yet produce crackers of good keeping quality, this is certainly not to be recommended as a general practice. A more detailed study must necessarily be made of the whole problem. The important fact derived from this study is that the keeping quality of a shortening as measured by storage tests or length of induction period of oxygen absorption may not be a true index of the keeping quality of the crackers baked from it.

Anti-oxygens

One phase of the rancidity problem which is receiving a great deal of attention at the present time is the study of anti-oxygenic catalysts. This study has followed three main lines of investigation: (1) A search for new anti-oxygens, (2) fundamental studies to account for the action of anti-oxygens, and (3) an attempt to determine the nature of anti-oxygens naturally present in fats.

A survey of the literature reveals numerous experiments to determine the applicability of substances as anti-oxygens for fats. Many of these substances which show such action have been patented and are finding commercial application in the preservation of fats for other than edible purposes. These anti-oxygenic substances vary considerably in structure although most of them used are aromatic amines or phenolic compounds. A partial list of these anti-oxygenic substances for fats which have been studied or patented the past few years would include the following: pyrogallol, pyrocatechol, α -naphthol, β -naphthol, β -naphthoquinone, hydroquinone, quinone, guaiacol, resorcinol, orcinol, phloroglucinol, thymol, o-cresol, p-cresol, eugenol, gossypol, α -naphthylamine, diphenylhydrazine, diphenylamine, diphenylguanidine, phenyl- α -naphthylamine, o-nitroaniline.

Due to the involved nature of the subject it would be impossible in a paper of this length to attempt to discuss the fundamental work rela-

tive to the theories of anti-oxygenic action and anyone desiring such information is referred to the original articles. A discussion of the early theories on anti-oxygens will be found in a paper by Moureau and Dufraisse (1926); later modifications are found in papers by Bäckström (1927), Mattill (1931), and Milas (1932).

Attempts to ascertain the nature of the substances responsible for anti-oxygenic activity in natural fats and oils dates from the work of Mattill (1927) in which he showed that wheat oil contained something which prevented the oxidation of vitamins A and E. This anti-oxygenic activity was considered by Mattill due to sterols present in the oil and this idea appeared to be substantiated later by the experiments of Mattill and Crawford (1930) in which it was demonstrated that sterols from corn oil prolonged the induction period of a lard-cod-liver oil mixture. Just which vegetable sterols are responsible for this action has not been determined due to the difficulty of their separation. Mattill (1931) found sitosterol, prepared from three different plant sources, without effect, as was also ergosterol and cholesterol.

Not all wheat oils necessarily possess anti-oxygenic activity according to Roller (1931), who found that some were totally inactive in this respect. He found the acetyl values of the active oils to be approximately 30 as compared to 15 for the inactive oils, and also concluded that the anti-oxygenic activity was connected with the -OH group.

Olcott and Mattill (1931) isolated and crystallized an anti-oxygenic material from lettuce which analyzed for a molecular weight of 250, and a possible formula of $C_{13}H_{14}O_5$. It was only slightly soluble in water and petroleum ether but soluble in ether, acetone, and dilute alkali. It would not add bromine, nor give a Libermann-Burchard test although an acetyl derivative could be formed. Using the autoxidation method of Mattill (1931) the substance had an anti-oxygenic index of 29 as compared to 31 for α -naphthol, indicating that it was a potent anti-oxygen.

Hilditch and Sleightholme (1932) have attacked the problem from a somewhat different angle. These investigators have attempted to determine the effect of the treatment of fats with certain reagents on the length of the induction period. They found, for example, that treatment with aqueous HCl or alkali almost eliminated the induction period in an olive oil sample and concluded from this that the natural anti-oxygenic substances present in this oil are water-soluble products readily removed by comparatively mild reagents. Treatment of the olive oil with sulphuric acid increased its resistance to oxidation indicating the possibility that the sulphuric acid is added at the double bond. While Hilditch and Sleightholme offer no suggestion as to the nature of the anti-oxygenic substances naturally present in fats and oils, they

make the pertinent statement that it is not likely that such anti-oxygens belong in all cases to the same class of substances.

A recent notice to the effect that Greenbank had discovered and patented a new anti-oxygen capable of being used in edible fats was recently reported in *Science* (News Supplement, Vol. 77, No. 1991, p. 6, 1933). In attempting to determine the anti-oxygenic substances naturally present in fats, Greenbank isolated extremely small amounts of unidentified organic acids. He then tried adding organic acids of known composition to oil and found that maleic acid added in the proportion of 1 part in 10,000 of oil would lengthen the keeping ability from 3-5 times. The mechanism of the reaction is unknown but the suggestion is made that it perhaps operates by stopping the formation of peroxides.

The structure of maleic acid [$\text{CO}_2\text{H}.\text{CH}:\text{CH}.\text{CO}_2\text{H}$] differs considerably from the previously noted anti-oxygens used in fats which were mostly organic amines or phenolic compounds. Since it is such a relatively simple compound as compared with the ordinary anti-oxygens, it should make an excellent substance for the study of the mechanism of anti-oxygenic action.

The report in *Science* on maleic acid unfortunately gives no details as to the method used for ascertaining the keeping ability of the oils treated with the anti-oxygen. We have determined the effect of maleic acid on the length of induction period of a few samples of open kettle and prime steam rendered lard and have found that maleic acid in concentration of 0.02% and 0.2% did not appreciably lengthen the induction periods of the lards studied when autoxidized at 90° without stirring. It is possible that in the case of some oils with longer induction periods, that the effect of maleic acid would become more pronounced since the report in *Science* indicates that Greenbank found "the better the quality of the oil the longer it would be preserved." It is also possible, of course, that the length of induction period as determined at 90° C. does not give a true picture of the anti-oxygenic effect of maleic acid and that storage tests at room temperature are necessary.

Lecithin has been suggested at various times as a possible anti-oxygenic substance in fats. Roller (1931), however, found that lecithin did not lengthen the induction period of oils or foods containing oils. We have obtained results similar to those of Roller on lecithin and also with choline derived from lecithin.

At the present we must consider that the exact nature of these anti-oxygenic substances is still unknown. The isolation by Bruson, Sebrell and Vogt (1927) of three natural oxidation inhibitors in rubber, two of which were sterols ($\text{C}_{27}\text{H}_{42}\text{O}_3$ and $\text{C}_{20}\text{H}_{30}\text{O}$) and one a phenolic compound illustrates the types of anti-oxygens possible in a plant material

such as rubber. The work of Mattill (1927), Mattill and Crawford (1930), Olcott and Mattill (1931) and Roller (1931) suggests the possibilities of sterol and phenolic compounds as anti-oxygens associated with the plant oils. Hilditch and Sleightholme (1932) found that the natural anti-oxygens present in the oils they examined were water-soluble substances. Greenbank appears to consider traces of organic acids as possible natural anti-oxygens and suggests maleic acid as such a substance. These results indicate that the natural anti-oxygens may belong to several types of substances and that they are likely sterols, phenols or organic acids.

The identification of the natural anti-oxygens present in fats and oils is a very important problem due to its commercial possibilities. A knowledge of the nature of these substances would be of great value for the intelligent preservation of these natural inhibitors during the processing of fats and oils for market. There would also be the possibility of adding natural substances to fats and foods without questionable physiological effects on the consumer or the prejudice against the addition of such things as chemical preservatives to natural food materials.

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FACTORS INVOLVED IN THE EXPERIMENTAL PRODUCTION AND PREVENTION OF HOCK DISEASE IN BATTERY BROODED CHICKS (*)

BY

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The increased use during the last few years of more intensive methods for the brooding and rearing of chicks has led to the increased occurrence of leg-bone deformities that are commonly referred to as Hock Disease, Slipped Tendons, Deformed Leg Bones or Perosis. This les weakness appears early in the chick's life, commonly during the third or fourth week but may occur at later periods. The first apparent symptom is a tendency for the chick to rest in a squatting position and as the condition is intensified, the legs of the afflicted birds present a bowed appearance and in many cases the hock joint becomes flattened. The tarso-metatarsi and tibiae become slightly bent, a condition which becomes more aggravated as the birds grow older. The tendon at the hock joint may slip from its condyles and produce a disabled joint after which the tendon usually becomes enlarged. The joints may or may not be filled with an excessive amount of fluid.

Hock Disease in its early appearance was confused with rickets by many investigators and attempts were made to combat the disorder by increased administrations of mineral and vitamin

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supplements. Since these attempts met with failure, investigations were conducted at several Experiment Stations to study the etiology of Hock Disease.

It has been definitely established by workers, at this and other Experiment Stations, that the abnormality is not rachitic in nature (1), (2), (3), (4), (5). The blood determinations as regards calcium and phosphorus and the bone findings so far as calcium, phosphorus and bone ash are concerned fall within the normal range. Histological examinations of the bones from hock diseased birds have revealed little regarding the cause or prevention of the disorder. The mineral elements in the ration and particularly the levels of calcium and phosphorus have been shown by several investigators to be contributing factors in the production of the abnormality.

EXPERIMENTAL

All investigational work reported in this paper was carried out with Single Comb White Leghorn chicks confined in battery brooders. The decks of the brooders were floored with one-half inch mesh wire screens and were so divided that each group of birds was confined to an area of approximately 720 square inches. The chicks were weighed, wing banded and placed in the catteries within a few hours after they were taken from the incubator. A maximum of thirty chicks were used in each group, although in some instances the groups were made up of a fewer number of individuals. Feed was placed before the chicks as soon as they were put in the batteries and kept before them at all times. The pens were so lighted that a twenty-four hour feeding period was afforded.

Under previous observations the basal ration composed of ground yellow corn 71, wheat middlings 5, alfalfa meal 3, dried skim milk 15, meat scrap 2 1/2, fish meal 2 1/2, and cod liver oil 1, in its supplemented form, did not produce Hock Disease in Single Comb White Leghorn chicks confined to battery brooders.

The basal ration, when supplemented with 2 % of bone meal, produced a large number of cases of Hock Disease but if 4 % of bone meal was added to the basal diet, a further increased number of cases occurred. Two per cent of bone meal in combination with 2 % of calcium carbonate, added to the basal ration, produced results almost as disastrous as when 4 % of bone meal was used. Four per cent of calcium carbonate added to the basal ration resulted in a high percentage of deformed birds. When 2 % of bone meal and 1.5 % of monobasic sodium phosphate were used in con-

bination, a large proportion of the birds became afflicted. The bone meal used was a good grade of steamed bone meal, the calcium carbonate was a finely ground limestone, and the sodium phosphate was of C. P. (chemically pure) quality. All supplements to the basal ration were made at the expense of the corn, and results are based on the number of birds alive at the end of the 8-week period. The following table (Table I) gives the results obtained when the basal ration was supplemented with various levels of the mineral supplements.

In other experimental work at this Station the observation was made that the occurrence of Hock Disease was lessened or prevented entirely when 20 parts of oat hulls replaced an equivalent amount of corn meal in the basal ration. Ground oat hulls, added to the ration at a level of 20 %, are likely to result in a reduced growth rate. Oat feed which is chiefly hulls but contains some oat middlings was found to be effective in the prevention of Hock Disease. A decreased growth rate was not obtained as was sometimes experienced when oat hulls were used. Ten per cent of oat feed when added to the basal diet was found to be insufficient to prevent Hock Disease when the basal diet contained 2 % of bone meal. Table II shows the effect of oat feed in preventing Hock Disease when varying amounts of bone meal are added to the basal ration.

DISCUSSION OF RESULTS

Examination of Table I will show that a large percentage of Hock Disease can be produced on a variety of levels and rations of calcium and phosphorus. Two per cent of bone meal added to the basal diet caused Hock Disease to appear in 56 % of the birds under observation. Four per cent of bone meal in the basal diet increased the number of deformed individuals to 88 %. Sodium phosphate and bone meal in combination while not essentially changing the calcium-phosphorus ratio from that of the unsupplemented basal ration, produced an average of 90 % of deformed birds as compared to none for the basal ration. Two per cent of calcium carbonate added to the basal ration produced 41 % of afflicted birds while a combination of 2 % of bone meal and 2 % calcium carbonate caused 90 % of the birds to show definite symptoms of Hock Disease.

Table II indicates that 20 % of oat feed or oat hulls was effective in preventing Hock Disease in the presence of either 2 or 4 % of bone meal in the ration. When 4 % of bone meal was added to the basal ration, 88 % of the chicks became affected

TABLE I. — *The effect of mineral supplements on the experimental production of Hock Disease.*

No. of pen	Additions to basal ration	No. of individuals	No. of birds afflicted	% afflicted	% Calcium in ration	% Phosphorus in ration	Ca : P ratio
101	None	25	0	0	.662	.587	1.13:1
124	None	20	0	0			
128	None	20	0	0			
132	None	20	0	0			
102	2% bone meal	23	13	57	1.262	.862	1.46:1
105	2% bone meal	21	13	62			
112	2% bone meal	19	11	58			
115	2% bone meal	23	12	52			
118	2% bone meal	22	11	50			
120	2% bone meal	29	18	62			
129	2% bone meal	25	15	60			
133	2% bone meal	19	12	63			
140	2% bone meal	29	13	45	1.861	1.137	1.64:1
Total		210	118	—			
Average per cent afflicted	56			
104	4% bone meal	24	20	83			
109	4% bone meal	22	20	91			
121	4% bone meal	29	27	93			
130	4% bone meal	23	20	87			
134	4% bone meal	19	16	84			
141	4% bone meal	28	24	86	1.262	1.195	1.06:1
Total		145	127	—			
Average per cent afflicted	88			
103	2% bone meal plus 1.5% sodium phosphate . .	22	20	91			
106	2% bone meal plus 1.5% sodium phosphate . .	24	21	88			
113	2% bone meal plus 1.5% sodium phosphate . .	19	16	84			
119	2% bone meal plus 1.5% sodium phosphate . .	24	23	96			
Total		89	80	—			
Average per cent afflicted	90	1.442	.582	2.48:1
111	2% calcium carbonate .	22	9	41			
110	2% bone meal plus 2% calcium carbonate .	25	22	88			
148	2% bone meal plus 2% calcium carbonate .	24	22	92			
Total		49	44	—	2.041	.857	2.38:1
Average per cent afflicted	90			

TABLE II. — *The effect of oat hulls and oat feed additions to the ration in the prevention of Hock Disease.*

No. of pen	Additions to basal ration	No. of individuals	No. of birds afflicted	% afflicted	% Calcium in ration	% Phosphorus in ration	Ca : P ratio
139	2% bone meal and 20% oat hulls	17	0	0	1.298	.892	1.46:1
137	4% bone meal and 20% oat hulls	16	0	0	1.898	1.157	1.64:1
108	2% bone meal and 20% oat feed	22	0	0	1.298	.892	1.46:1
114	2% bone meal and 20% oat feed	20	0	0			
116	2% bone meal and 20% oat feed	24	1	4			
122	2% bone meal and 20% oat feed	28	1	4			
125	2% bone meal and 20% oat feed	20	0	0			
138	2% bone meal and 20% oat feed	19	0	0			
142	2% bone meal and 20% oat feed	30	0	0			
Total		163	2	—			
Per cent afflicted	1	1.898	1.157	1.64:1
136	4% bone meal and 20% oat feed	19	1	5			
146	4% bone meal and 20% oat feed	24	0	0			
Total		43	1	—			
Per cent afflicted		—	—	2	(c)	(c)	(c)
123	2% bone meal and oat feed ash (a)	27	11	41			
117	2% bone meal and Ext. oat feed (b)	24	11	46			
147	2% bone meal and Ext. oat feed (b)	25	13	52			
Total		49	24	—	49		
Per cent afflicted	49			

(a) Oat feed charred and added to the ration in amount equivalent to 20% of the original oat feed.

(b) Oat feed digested with weak acid and alkali and added to the ration in amount equivalent to 20% of the original oat feed.

(c) Undetermined.

but when 20 parts of oat feed replaced 20 parts of corn the condition was reduced to 2 %. When 2 % of bone meal was added to the basal diet 56 % of the birds were afflicted but when a further addition of 20 parts of oat feed was made, the trouble was reduced to 1 %. TITUS (5) has found that rice bran exerts a protective action against Hock Disease and that 10 % of this material, under his experimental conditions, was quite effective. Obviously the feeding of such materials as rice bran or oat feed has little effect on the calcium and phosphorus levels of the ration. Since there was the possibility that the fiber in the oat hull was the preventive agent, this possibility was excluded by extracting quantities of oat feed with dilute acid and alkali. The feeding of the extracted residue in an amount equivalent to 20 % of the original material, showed that oat feed thus treated no longer possessed the property of preventing Hock Disease in the basal ration in the presence of 2 % of bone meal. It is not probable that the beneficial effect of oat feed is due to ash ingredients because the feeding of charred oat feed, equivalent to 20 % of the original material, showed that it had lost its power for preventing Hock Disease. Research work under way at the present time indicates that the beneficial effect of oat feed may be due to its physical properties.

CONCLUSIONS

Hock Disease in battery brooded Single Comb White Leghorn chicks can be caused or prevented by mineral adjustments in the ration. The abnormality can be produced by additions to the basal ration of calcium or phosphorus or both and can be brought about under a variety of calcium-phosphorus ratios. Finely ground oat hulls possesses some property of prevention of Hock Disease even under unfavorable mineral conditions. This property cannot be explained on the basis of the fiber or ash content of the hull.

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1933, xxxi, pp. 70-75

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Relative Utilization of Calcium from Calcium Carbonate and Calcium Gluconate by Chickens.

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Numerous investigations have indicated that the chicken may use a number of forms of calcium for its metabolic needs. Workers at the Kentucky Experiment Station¹ studied calcium carbonate, calcium lactate, calcium sulphate, tri-calcium phosphate and calcium chloride as sources of calcium for chickens and found that calcium carbonate was the most effective judged by the degree to which it was utilized in the production of eggs, its influence on the weight of egg contents and shells, and quantity of the salt consumed. Calcium sulphate was not so effective as the carbonate as shown by a smaller egg production and lower weight of shells and egg contents. Calcium lactate was readily utilized but the quantity consumed was variable and small as compared to the carbonate and sulphate. Only small quantities of calcium chloride were consumed and the precipitated tri-calcium phosphate was not a satisfactory source of calcium for egg production as compared to calcium carbonate. The above mentioned salts were fed as supplements to a wheat, yellow corn and skim milk ration.

Bethke² and his associates state that no difference was found in the availability of calcium in the carbonate, sulfate, lactate and phosphate salts when fed to chicks on a basis of equal calcium intake on a minimum requirement basis.

As calcium gluconate is a readily soluble salt, it was thought desirable to study it as a source of calcium as compared to calcium

¹ Buckner, G. D., Martin, J. H., and Peter, A. M., *J. Agr. Res.*, 1928, **36**, 263.

² Bethke, R. M., Kennard, D. C., and Kick, C. H., *Poultry Science*, 1929, **9**, 45.

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carbonate, since the carbonate is the commonly used source of supplemental calcium for poultry. The following studies include both growing chicks and laying hens.

Two groups each consisting of 81 day-old Barred Rock chicks were placed in battery brooders and given rations that differed only as to the source of calcium. Group I received calcium carbonate as its supplemental source of calcium, while Group II received calcium gluconate.* These salts were incorporated with the ration which was fed in all-mash form and kept before the birds at all times. Table I gives the make-up of the starting and growing ration as well as the ration used for the laying birds in the second part of the experiment.

TABLE I

	Starting and growing ration		Laying ration	
	Ration I	Ration II	Ration I	Ration II
Ground yellow corn	34.5	35.2	27.9	29.46
" wheat	15	15	15	15
" oats	20	20	20	20
Alfalfa meal	10	10	10	10
Meat meal	5	5	5	5
Fish "	5	5	5	5
Dried Milk	5	5	5	5
Calcium carbonate	1		2.2	
" gluconate		4.32		9.5
Sodium phosphate			.8	.8
Commercial sucrose	4		8.9	
Cod liver oil	.5	.5	.2	.2
	100	100	100	100
% calcium	1.38	1.40	1.86	1.88
% phosphorus	0.657	0.662	0.848	0.851

The sugar was added to the calcium carbonate rations to compensate for the gluconic acid carried by the gluconate rations.

The males were discarded at the end of 6 weeks, and at 8 weeks of age, 8 pullets were removed from each group, the left tibia was dissected from each and bone ash determinations made on the oven dry fat-free tibiae. Group I exhibited an average bone ash of 49.8%, Group II a bone ash value of 48.9%. Since vitamin D had been supplied in ample quantity it was thought advisable to study the bone ash of birds that had been fed the same rations as were fed to Groups I and II but to restrict the cod liver oil to 0.10% of the total ration. Previous observations had shown that when this level of cod liver oil was administered, many birds became rachitic at an early age. Two groups of 15 chicks each were used in this

* The calcium gluconate used in the study was obtained through the courtesy of Röhm and Haas Co., Inc., of Bristol, Pa.

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study and when bone ash determinations were made at 8 weeks, the birds receiving calcium carbonate had an average bone ash of 37.6 and the ones receiving calcium gluconate had an average bone ash of 39.2. No further studies were made with these birds.

When the birds that were started on the original experiment were 22 weeks old, they were taken from the batteries and 12 birds from each group were placed in individual compartments of laying cages. At this time the average weights of the birds in Groups I and II were 1,450 gm. and 1,535 gm., respectively. The results with growing chicks are in agreement with those of Hart³ and associates, who compared calcium carbonate, calcium sulfate, bone meal, rock phosphate, dicalcium phosphate and calcium gluconate as sources of calcium for the growing chick in the presence of ample quantities of vitamin D. They state that: "These findings indicate clearly that it cannot be assumed that one form of calcium is better than another for animal feeding because it is more soluble in water. The rate of solution of calcium salts in the animal intestine is sufficient, even in case of the more insoluble compounds to make all forms equally effective to the animal provided adequate vitamin D is present."

The rations fed to the birds after they were placed in the laying cages differed only slightly from the rations they received during the growing period. It was necessary, however, to add more calcium to the ration to take care of the increased demand for calcium for the production of egg shells. The phosphorus content of the rations was also raised, by the addition of sodium phosphate. It was felt that in this study any differences that might result from the use of the 2 salts would be more apparent if vitamin D were supplied in limited amounts, consequently 0.2% of cod liver oil was supplied in the ration which was fed as all mash and kept before the birds at all times. This amount of cod liver oil furnished vitamin D in quantity less than that required for good egg production from birds denied access to sunlight. The laying birds in this experiment were housed in a basement room and any sunlight reaching them was filtered through window glass. The composition of the ration used for egg production is given in Table I.

After egg production started the following data were collected: number and weight of eggs produced, weight of shell, weight of shell ash, and in some cases the calcium level of egg contents was determined. Limited studies were also made on the hatchability of

³ Hart, E. B., and Deobald, H. J., Wisconsin Bulletin 421.

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eggs and livability of the resultant chicks. The birds continued on the study for 12 months after egg production started and egg shell studies were made except for 2 months during which time the eggs were used for hatchability studies. The determination of calcium in egg contents was not started until after the birds had been in production for 3 months and was discontinued while the eggs were being used in hatchability studies.

Eggs were collected daily and were weighed the following day, at which time the shells were removed, washed free from adhering contents, dried to constant weight and ashed. For the most part the shells from a number of eggs were pooled for the ash determinations. Table II gives the shell and shell ash determinations by months, the percentages being based on the weights of the original eggs. Table II also includes results of calcium determination on egg contents.

TABLE II

Month	Shell		Shell Ash		Ca in egg contents	
	Group I %	Group II %	Group I %	Group II %	Group I %	Group II %
1	7.9	8.8	4.1	4.7		
2	8.3	8.5	4.3	4.5		
3	7.6	8.5	3.9	4.4	.051	.053
4	8.0	8.9	4.1	4.5	.050	.053
5	8.1	9.0	4.3	4.7	.051	.053
6	8.0	9.1	4.2	4.8	.050	.053
7	8.3	8.8	4.5	4.7	.050	.052
10	8.4	8.9	4.6	4.9	.050	.052
11	8.3	8.8	4.4	4.6	.050	.052
12	8.3	8.7	4.3	4.5	.051	.052
Average	8.13	8.79	4.27	4.62	.050	.053

Calcium determinations were made on pooled samples of eggs from the 2 groups and in order to obtain a representative sample, the eggs were broken and stirred for 10 minutes with an electric stirrer before samples were taken for analysis. The contents of eggs produced by Group I had an average analysis of .050% calcium and the eggs produced by Group II had an average of .053% calcium for the 8 months during which these determinations were made.

Since it was observed that the calcium level of the egg contents was somewhat higher in the group receiving calcium gluconate, it was thought desirable to determine the relative amounts of thick and thin albumen present in the eggs produced by the 2 groups of birds. This was done using the method and apparatus described by Holst and Almquist.⁴

⁴ Holst, W. F., and Almquist, H. J., *Hilgardia* 6, 48, 1931.

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Group I produced an average of 105 eggs per hen during the year, with an average weight of 50.1 gm. per egg, while Group II in the same time produced an average of 115 eggs with an average weight of 50.5 gm.

Seven birds out of the 12 in Group I that were started in the second part of the experiment survived and 8 birds of the 12 that were started in Group II were alive at the completion of the study. All hens were individually mated and observations made as to the fertility and hatchability of eggs produced by the 2 groups. The fertility in both groups was quite low but no difference between the groups was noted. The hatchability of fertile eggs was satisfactory for both groups. Because of the small number of birds involved, the hatchability data are not presented.

In the presence of ample vitamin D, calcium gluconate seems to function as well as calcium carbonate as a source of calcium for the growing chick. The same is true when these supplements are supplied in rations where vitamin D is limited. The birds receiving calcium gluconate grew slightly better than those receiving calcium carbonate. In rations fed to the laying hen, where vitamin D was supplied in quantity less than the optimal, calcium gluconate appears to be somewhat more efficient than calcium carbonate in the formation of egg shell, as evidenced by greater weight of egg shell and a higher percentage of shell ash. Calcium gluconate under the conditions involved in these experiments also caused a greater deposition of calcium in the inner parts of the egg. It has been generally believed that the calcium content of the interior parts of the egg is constant and not influenced by feeding or other treatment of the hens, but Hughes and associates⁵ were able to raise the calcium level of the egg contents by irradiating the hens producing the eggs.

During the first few months that observations were made on the amount of thick white present, it appeared that the eggs produced on the calcium gluconate ration had a higher percentage of thick white than those produced on the calcium carbonate ration. This difference tended to lessen as the study proceeded and the average percentage of thick whites from the 2 groups was not substantially different when calculated for the entire period.

Due to differences in the calcium content of the 2 salts studied, it was necessary to use more than 3 times as much gluconate as carbonate to maintain an equal calcium level. The use of calcium

⁵ Hughes, J. S., Payne, L. F., and Latshaw, W. L., *Poultry Science*, 1923, 3, 151.

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gluconate in such amounts as were used in this study is likely to be impractical from a cost standpoint, but it would be desirable to determine whether or not the gluconate has a supplemental effect when the major part of the calcium in the ration is furnished by calcium carbonate or some other source of calcium. The level of vitamin D was decidedly lower than the optimum level and it is possible that the birds could function as well as they did under the conditions of this experiment, if lower levels of calcium were used in the presence of ample vitamin D.

The number of birds involved in the egg production studies was quite small but the differences observed in shell, shell ash and calcium level of the egg contents seem to be significant.

Summary. Calcium carbonate and calcium gluconate seem to function with equal efficiency as sources of calcium for the growing chick when equivalent amounts of calcium are supplied. With the laying hen the calcium gluconate seems to function slightly more efficiently than the carbonate, as evidenced by increased egg shell, shell ash, and an increase of calcium in the egg contents.

ABSORPTION, UTILIZATION, AND RECOVERY OF NITROGEN, PHOSPHORUS, AND POTASSIUM BY APPLE TREES GROWN IN CYLINDERS AND SUBJECTED TO DIFFERENTIAL TREATMENT WITH NUTRIENT SALTS

BY

WALTER THOMAS

(Contribution from Pennsylvania Agricultural Experiment Station)

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ABSORPTION, UTILIZATION, AND RECOVERY OF NITROGEN, PHOSPHORUS, AND POTASSIUM BY APPLE TREES GROWN IN CYLINDERS AND SUBJECTED TO DIFFERENTIAL TREATMENT WITH NUTRIENT SALTS¹

By WALTER THOMAS

Professor of phytochemistry, Pennsylvania Agricultural Experiment Station

INTRODUCTION

In a recent paper the writer (32)² presented data on the composition of the season's branch growth at successive periods of the growth cycle in relation to the vegetative and reproductive responses of apple trees subjected to treatment with different combinations of nutrient salts, when grown in metal cylinders. The present paper reports the total amounts of nitrogen, phosphorus, and potassium (as N, P₂O₅, and K₂O) absorbed during the whole 6-year growth period by a representative tree under each treatment.

The trees in each of the two culture systems—sod and tillage—in this experiment were exposed to identical external conditions of temperature, rainfall, light, and heat, and in addition to a uniform soil substrate (26). Considered, therefore, as separate systems, the only variables to which the trees under sod and tillage, respectively, were subjected, therefore, were the mineral nutrient salts added.

A voluminous literature on the absorption and utilization of the principal nutrient elements by plants is extant. The writer (27, 28, 29, 30, 31) has critically discussed and summarized our present knowledge of this subject with reference especially to the absorption of nitrogen, phosphorus, and potassium, and Hoagland (9, 10) has reviewed the more recent (1930-31) contributions. According to the views of Steward (24) and Hoagland (9) the process of absorption requires the expenditure of energy, the source of which is metabolic, determined by oxygen absorption or limited by carbon dioxide accumulation, the ultimate mechanism being determined by the electromotive forces brought about by oxidation-reduction potentials.

PREVIOUS INVESTIGATIONS ON THE TOTAL AMOUNTS OF NITROGEN, PHOSPHORUS, AND POTASSIUM REMOVED BY APPLE TREES

The object of the early experiments on the amounts of nitrogen, phosphorus, and potassium absorbed by the Pomaceae was to acquire information relating to the proximate amounts of these elements annually withdrawn from the soil by mature trees of maximum reproductive capacity.

Roberts (20) investigated a 13-year-old Wagener apple tree. The composition of the leaves sampled in the fall with respect to ash, nitrogen, phosphorus, and potassium was established. From these

¹ Received for publication Mar. 28, 1933; issued October, 1933. Technical Paper no. 597 of the Pennsylvania Agricultural Experiment Station. Presented before the joint sessions of the American Societies of Horticultural Science and Plant Physiologists at the Atlantic City meeting of the American Association for the Advancement of Science, 1932.

² Reference is made by number (italic) to Literature Cited, p. 579.

analyses and the average composition of apples obtained from another source, Roberts, by making numerous assumptions, presented an approximate estimate of the amounts of these nutrient elements removed by fruit and leaves at various ages of the tree.

Ten years later Van Slyke and his coworkers (34) reported the amounts of nitrogen, phosphorus, and potassium in the season's growth of a Baldwin and also of a Rhode Island Greening apple tree. The trees were about 30 years old. The approximate annual average removal of nutrients from the soil by trees of this age is reported as nitrogen, 1.5 pounds; phosphoric acid, 0.4 pounds; and potash, 1.6 pounds.

During the next decade Thompson (33) planted in the experimental plots of the Arkansas Agricultural Experiment Station seedlings obtained from five different known varieties of apple (average weight of trees 117 g). At the end of the growing season of each year the nitrogen, phosphorus, and potassium content of two trees of each variety were determined. Thompson reported the climatic conditions and also the analyses of the soil but did not mention either the fertilizer treatments (if any) or the culture system under which the trees were grown. Reference will be made later to certain of his data.

PLAN OF THE EXPERIMENT

Inasmuch as the plan of the present experiment has been given in detail in previous publications (1, 32), it will only be necessary to recapitulate briefly. In May 1922, stocks representing a single clone were obtained from the East Malling Research Station, England, and were planted in a Hagerstown silty clay loam soil. Two weeks later they were whip-grafted to Stayman Winesap scions from a tree whose performance was known for two-bud generations (1). Boiler-plate cylinders 5 feet in diameter and 5½ feet deep were filled with the soil previously thoroughly mixed with respect to each of the three soil horizons (26, 32). Three years after planting, the trees (six in each row) were subjected to differential nutrient treatments by the application of various combinations of the following pure salts: NaNO_3 , $\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$, and K_2SO_4 . The combinations added were (1) $\text{NaNO}_3 + \text{CaH}_4(\text{PO}_4)_2 + \text{K}_2\text{SO}_4$ (symbol NPK); (2) $\text{NaNO}_3 + \text{CaH}_4(\text{PO}_4)_2$ (symbol NP); (3) $\text{NaNO}_3 + \text{K}_2\text{SO}_4$ (symbol NK); (4) $\text{CaH}_4(\text{PO}_4)_2 + \text{K}_2\text{SO}_4$ (symbol PK); (5) NaNO_3 (symbol N); (6) $\text{CaH}_4(\text{PO}_4)_2$ (symbol P); (7) one row of trees received no nutrient salt addition (symbol check).

Half of the trees under each treatment were maintained in a timothy and bluegrass sod, and the other half were subjected to a tillage system. The practical field details and also the growth records during the experiment period under each system have been given by Anthony and Clarke (1). In October 1927, a representative tree growing under (1) the sod, and (2) the tillage systems, respectively, in each of the nutrient salt treatments was removed and divided for purposes of chemical analyses into (1) leaves, (2) trunk, (3) large branches, (4) small branches, (5) large roots, (6) small roots, and (7) fruit (if any).

Representative samples of the portions so separated were secured for analysis by first cutting them into small pieces, and then reducing by the process of successive quartering used in ore sampling. The

final samples representative of each division were dried at 100° F. and then ground in a Wiley mill to pass a 100-mesh sieve. Because of the difficulty in removing all the attached soil by washing, the results for the roots are calculated to a silica-free basis. The analytical methods adopted were identical with those previously described (32).

EXPERIMENTAL DATA

The analytical results are summarized in table 1. The detailed analyses are omitted, but investigators desiring the detailed analyses of the grafted Stayman Winesaps and of the trees when dug up and also of the prunings may procure them from the research office of the School of Agriculture of this institution or from the author. Photographs of six of the trees grown in sod are given in figures 1 and 2. Photographs of some of the other trees appear in the paper by Anthony and Clarke (1).

TABLE 1.—Total nitrogen, phosphorus, and potash (grams) removed by trees from cultivated and uncultivated soil, to which different mineral treatments were applied, during entire growth period, 1922–27

Treatment	Dry weight of trees (grams)		Total N absorbed during growth		Total P_2O_5 absorbed during growth		Total K_2O absorbed during growth	
	Under cultivation	In sod	Under cultivation	In sod	Under cultivation	In sod	Under cultivation	In sod
Check.....	12,228	8,886	35.8	18.8	15.1	10.6	40.5	28.7
NPK.....	11,792	15,265	136.8	142.8	26.1	26.6	80.7	81.4
NP.....	16,005	15,607	129.6	138.2	23.1	25.2	71.4	65.3
NK.....	14,504	15,668	96.5	96.7	18.5	19.9	69.0	76.6
PK.....	8,996	8,326	39.8	28.6	13.0	13.1	41.5	33.8
N.....	12,109	13,053	83.3	81.0	15.3	16.4	51.1	56.6
P.....	12,239	7,595	53.1	31.2	17.1	11.5	50.1	29.5

INCLUDING LEAVES								
Check.....	14,797	11,433	53.5	35.4	17.6	12.9	65.1	52.1
NPK.....	18,365	18,891	180.9	187.7	31.6	32.1	110.4	136.9
NP.....	19,277	18,975	170.3	180.3	27.8	29.9	109.3	99.6
NK.....	17,679	18,430	131.8	127.3	22.6	23.5	114.4	113.4
PK.....	11,788	11,054	63.4	55.0	15.8	17.3	79.7	71.1
N.....	15,419	16,139	121.3	119.0	19.9	20.7	90.9	93.0
P.....	14,935	9,907	77.1	47.9	29.4	14.0	79.8	51.9

INTERPRETATION AND DISCUSSION OF RESULTS

The distinguishing feature of the results is the great differences in the assimilation of nitrogen, phosphorus, and potassium resulting from the different nutrient salt treatments.

In considering the effect of these different treatments on carbohydrate metabolism, it has been found (32) that the concentration of carbohydrates in the season's growth follows the ascending series $\text{NPK} < \text{NP} < \text{NK} < \text{N} < \text{PK} < \text{P} < \text{check}$, showing that carbohydrates have accumulated in much larger amounts in the trees which did not receive nitrogen additions. The relatively small absolute amounts of nitrogen, phosphorus, and potassium found (table 1) in the trees receiving no nitrogen is an indication that the low concentrations of



FIGURE 1.—Trees grown in sod: A, no. E 1, treated with monocalcium phosphate only (symbol P); B, no. E 2, treated with sodium nitrate only (symbol N); C, no. E 3, treated with sodium nitrate, monocalcium phosphate, and potassium sulphate (symbol NPK).

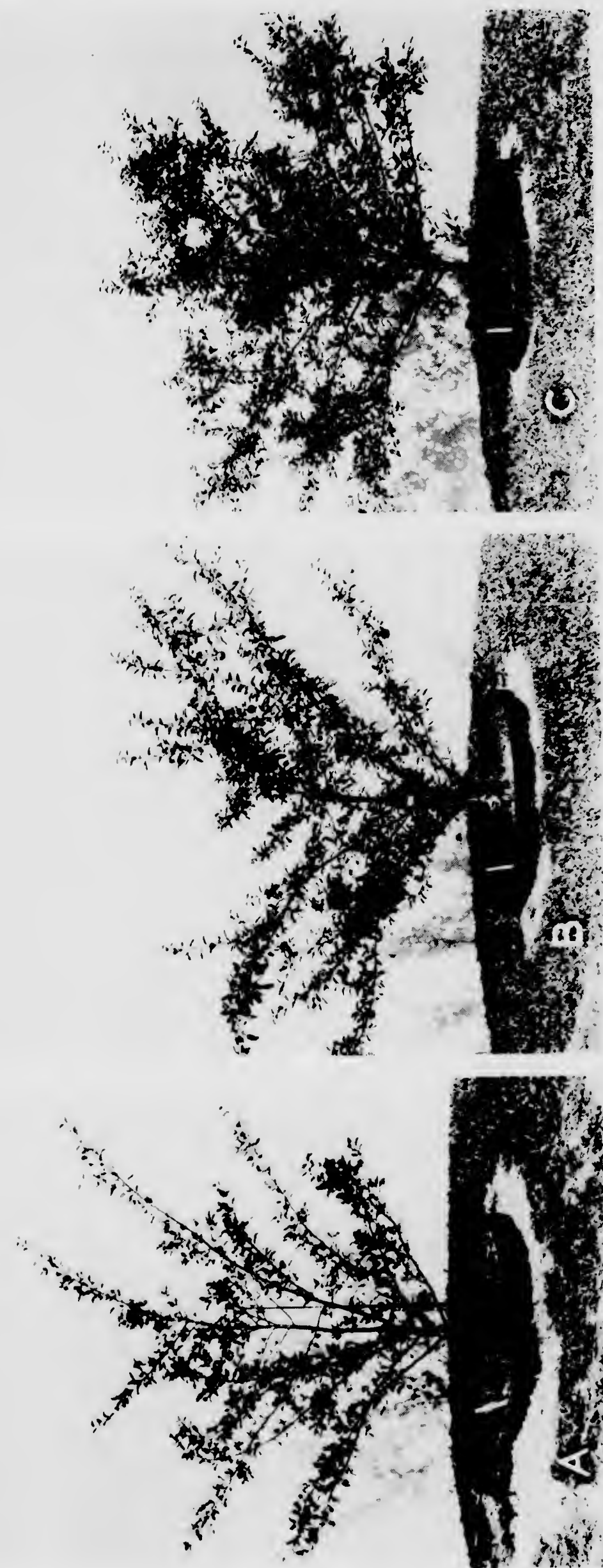


FIGURE 2.—Trees grown in sod: A, no. E 4, untreated check; B, no. E 5, treated with monocalcium phosphate and potassium sulphate (symbol PK); C, no. D 7, treated with sodium nitrate and monocalcium phosphate (symbol NP).

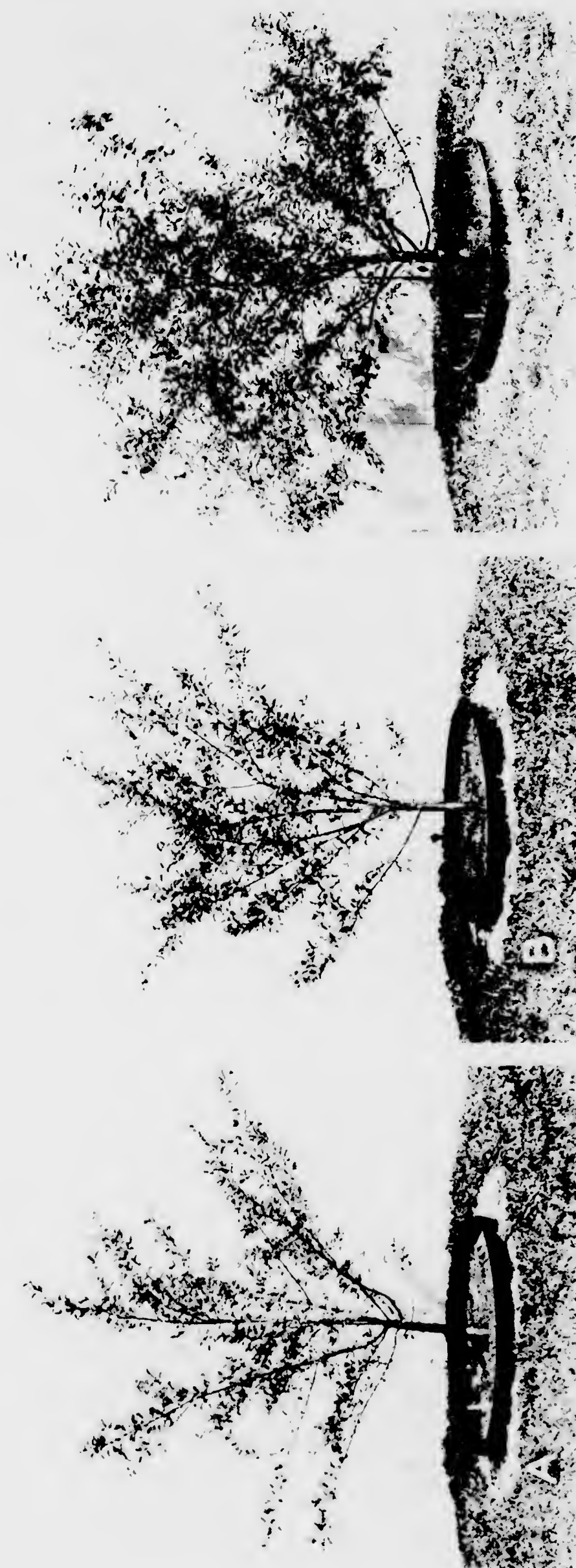


FIG. 1. 1. Trees grown in soil. A, no. F. 1, treated with monocalcium phosphate only (symbol P); B, no. F. 2, treated with sodium nitrate, monocalcium phosphate, and potassium sulphate (symbol NPK); C, no. F. 3, treated with sodium nitrate, monocalcium phosphate, and potassium sulphate (symbol NPK).

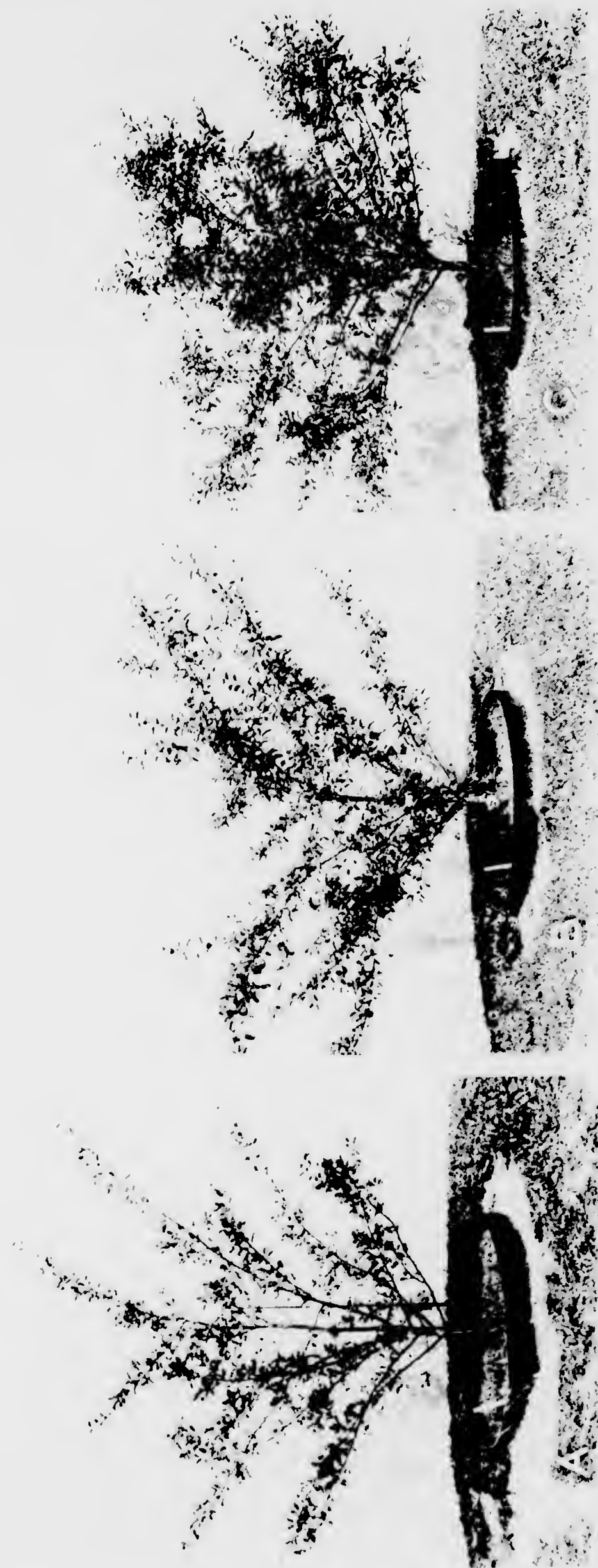


FIG. 2. 1. Trees grown in soil. A, no. F. 4, untreated check; B, no. F. 5, treated with monocalcium phosphate and potassium sulphate (symbol PK); C, no. F. 6, treated with sodium nitrate and monocalcium phosphate (symbol NP).

these elements calculated earlier (32) on the basis of percentage of dry weight of the season's branch growth is the result of a much lower intake of these elements and not a masking effect caused by the relatively high amounts of carbohydrates.

The concentration of nitrogen in the season's growth has been shown to follow the ascending series check $<P < N < NK < PK < NPK$ (32). This order closely parallels the data for the total growth of the trees under each of the respective nutrient treatments. These results explain why the nitrogen factor plays such a dominant role in fruit growing.

SPECIFIC EFFECT OF EACH OF THE PRINCIPAL ELEMENTS

The writer has summarized (28, 29, 31) the results of field experiments which supply data on the effect produced, if one of the principal nutrient elements (nitrogen, phosphorus, or potassium) is omitted from a fertilizer. In some of these experiments (13) the omission of one of these elements resulted in an increased absorption of the other elements of the fertilizer with concomitant decrease in growth and yield of fruit attributed to a nutritional lack of balance. But in other field experiments (23, 31) the omission of an element from a fertilizer containing all three elements (nitrogen, phosphorus, and potassium) resulted in a decreased absorption of the other elements present. An explanation of such anomalous results has already been given by the writer (31).

The results summarized in table 1, which shows the amounts of nitrogen, phosphorus, and potassium (as N, P_2O_5 and K_2O) absorbed by the trees during the entire growth period of 6 years, indicate that the omission of one of these elements from a fertilizer has resulted in a decreased absorption and assimilation of the elements present in the incomplete fertilizer as compared with the complete fertilizer (cf. the amounts absorbed by the trees which received two nutrient elements, viz. NP, NK, and PK, with the absorption by the trees treated with all three nutrients, NPK). Such results are in accordance with Liebig's law of the minimum. This decreased absorption resulted in a lack of nutritional balance characterized by decreased growth and blossom formation except in the NP treatment, in which a decreased absorption of potassium resulted in slightly increased growth and blossoming.

In attempting to isolate the specific effect of an element in relation to the energy transformation in any living system, it is necessary to keep in mind the fact that the Wirkungswert (effect factor) of an element may not be (and generally is not) the same in the presence of another factor or factors as when the factor operates alone. Experimental results may be cited in which, when two factors vary simultaneously, each may produce its own effect independently of the other, as in Mitscherlich's experiments (16, 22). Mitscherlich found that when oats were grown in sand receiving varying amounts of water and calcium phosphate the relative effects on the yield produced by the different amounts of water were constant however much phosphate was added. But since numerous investigators (5, 7, 14, 19) have been unable to confirm the constancy of Mitscherlich's Wirkungswert for each fertilizer, the general principle enunciated in the opening of this paragraph must be accepted.

The data of the present experiment show that the amount of an added element absorbed by a plant and its effect on growth and reproduction is a function of the elements with which it is associated. The plant has a capacity to grow in a manner that cannot be regarded as a simple process of addition and subtraction of single effect factors. It is with these limitations in mind that the absorption and recovery of elements are discussed. An interesting and lucid explanation of the operation of growth factors is given by Nolte (17).

EFFECT OF THE OMISSION OF NITROGEN

Table 2 shows the amounts by which the absorption of each of the elements has been reduced by the omission of nitrogen and the quantitative effect of this reduced absorption in reducing growth and blossoming. The values given in columns 2 to 7 represent the difference in the amounts of the elements absorbed in each of two comparisons from which nitrogen has been omitted.

TABLE 2.—Effect of the decreased absorption^a of each of the nutrient mineral elements in reducing growth and blossoming following the omission of nitrogen, phosphorus, or potassium

Treatments compared	NITROGEN OMITTED									
	N		P_2O_5		K_2O		Growth		Blossoms removed to thin to 6 inches apart, 1925-27	
	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod
	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Number	Number
NPK and PK	117.6	132.7	15.7	14.7	60.6	55.7	6,577	7,836	530	727
NP and P	93.3	32.5	7.4	15.9	29.5	44.6	4,311	9,068	689	699
	PHOSPHORUS OMITTED									
	N		P_2O_5		K_2O		Growth		Blossoms removed to thin to 6 inches apart, 1925-27	
	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod
	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Number	Number
NPK and NK	49.2	60.4	8.9	8.6	25.9	23.5	686	461	272	348
NP and N	48.9	61.5	7.8	9.3	18.5	3.6	2,858	2,836	679	576
	POTASSIUM OMITTED									
	N		P_2O_5		K_2O		Growth		Blossoms removed to thin to 6 inches apart, 1925-27	
	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod
	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Number	Number
NPK and NP	10.7	7.3	3.8	3.2	31.0	37.3	911	84	195	28
NK and N	10.4	8.2	2.7	2.8	31.6	58.4	2,261	2,291	212	256

^a Differences, in grams, in quantities of each element absorbed are shown in columns 2 to 7.

^b In these cases only, an increase and not a decrease occurred.

The data in table 2 indicate that no simple quantitative relationship exists between the amounts by which the absorption of each of the elements have been reduced as a result of the omission of nitrogen from the fertilizer and the accompanying reduction in growth and blossoming. The effect values of nitrogen applied together with phosphorus are not the same when potassium is present as when that element is absent. Moreover, this effect changes from a negative to a positive value in the trees under cultivation and sod, respectively.

EFFECT OF THE OMISSION OF PHOSPHORUS

Table 2 also shows the amounts by which each of the elements has been reduced as a result of the omission of phosphorus from the fertilizer and the accompanying disturbance in metabolism shown by the extent to which growth and blossoming have decreased. The values given in the table express the difference in the amounts of the elements absorbed in each of two comparisons from which phosphorus has been omitted.

The table is of interest because it shows that the quantitative effect of the omission of phosphorus on the reduction of growth and blossoming is much greater in the absence of potassium than when this element is present. In other words, the effect factor for phosphorus when applied together with nitrogen is much greater if no potassium is added. Since the differences in the amounts of nitrogen absorbed under the respective culture systems in both comparisons are practically identical, one must attribute the marked effect of the omission of phosphorus in the present experiments to differences in the amounts of potassium absorbed.

EFFECT OF THE OMISSION OF POTASSIUM

Table 2 further shows the quantitative effect of the omission of potassium on the reduction in the absorption of each of the elements and the effect of this reduced absorption on growth and blossoming.

The omission of potassium resulted in increased growth in the combination NP, as compared with NPK, both under cultivation and in sod, and also in increased blossoming under cultivation. An explanation of this anomalous behavior will be apparent later from the discussion (p. 576) of the ratios in which the elements have been absorbed.

UTILIZATION AND RECOVERY OF NITROGEN, PHOSPHORUS, AND POTASSIUM

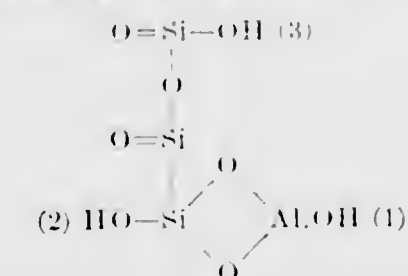
Theoretically a distinction must be drawn between the amount of an element absorbed and the amount utilized by the plant. Some authorities (11) claim that the absorbed but unutilized portion of a nutrient element is unchanged and is found as dissociated inorganic salts in the vessels and cell sap, and, although critical experiments on the subject have yet to be conducted, the fact that the results of many investigators (2, 23, 35) indicate that at least in some plants the portion of an element absorbed in excess is expelled via the roots shortly after blossoming time, would lend support to this view. If Jacob's views (11) are correct, absorption and the utilization of an element as determined by the analysis of a plant at maturity would be synonymous. Some of the results of this experiment, however, do not conform to this concept.

Present usage of the terms "absorption", "assimilation", "utilization", and "recovery" are somewhat confusing. In the present paper the term "recovery of an element" will be used to indicate that portion of an element applied as fertilizer which has been absorbed by the plant.

The amount of an element absorbed is determined, among other factors, not only by the species of plant and the availability of the fertilizer per se, but also by the indirect effects produced by the interaction of the fertilizer (or salt) with the components of the solid and liquid phases of the soil and also by interionic effects. These

effects, which may be very complex, are manifested in the so-called "phenomena of antagonism" and in the disturbance of equilibrium through displacement of bases in the soil colloidal complex by one or more of the elements in the fertilizer, and in the phenomenon of fixation by the iron and aluminum of the aluminosilicates as well as by the microorganisms—the so-called "biological absorption" (25). These effects were very marked in the present experiment.

In the clay soils of the type used in the present experiment the outer hydrophilic coatings of the clay particles dominate the soil system and, inasmuch as the majority of soils give rise to negative aluminosilicate ions, low results for the recovery by the plant of cations may in part be explained on the basis of adsorption. But the explanation of the low recovery of anions such as the phosphate ion by such a similar fixation is more difficult to explain, unless the explanation of some investigators (12) be accepted, of the existence of a Helmholtz triple layer, or of Clarendon (3) of a clay structure represented by the constitutional formula



in which the basic hydroxyl group (1) attached to the aluminum ion can be replaced by acid radicals.

RECOVERY OF NITROGEN

For reasons already given the recovery of an element from an added fertilizer cannot be determined with exactness in field experiments. In a controlled experiment of the present type (each cultural system considered separately), in which the trees have been subjected to the same growth factors except the addition of mineral salts, the values may be calculated accurately enough for practical purposes.

The values which represent the amounts of the respective elements absorbed from the added fertilizers given in tables 3, 4, and 6 were obtained from the difference between the amount of the element absorbed by the respective check tree and that of the treated tree under examination. This method gives values for the recovery somewhat higher than those that would be obtained by using instead of the check tree the tree which has received all the elements except the one of which the recovery is to be determined. The values for the recovery in the tables may thus be said to be upper limits.

In grain and vegetable crops the recovery of added nitrogen by the plant averages about 50 percent (15, 18, 21), but no data appear to be available on the recovery of nitrogen by fruit trees. In solution cultures at very high concentrations (0.02 to 0.16 percent) (8), the absorption-coefficient for the nitrate ion is very high, reaching 100 percent in the higher concentrations. The relatively low recovery by the plant of added nitrates in field experiments may be attributed not only to the low absorption-coefficient resulting from the relatively dilute concentration of the soil solution but also to the losses by leaching, to processes rendering the nitrogen immobile, and also (especially under cultivation) to losses as gaseous nitrogen.

The total amount of sodium nitrate applied to each of the trees receiving nitrate was 2,824 g, equivalent to 464.5 g calculated as elemental nitrogen. This corresponds to an annual application of 154.8 g, or to 0.33 pound of nitrogen per tree. The current practice in the experimental orchards of this station is to apply 5 pounds of sodium nitrate, equivalent to 0.8 pound of nitrogen, to mature trees, much larger than those used in the cylinders.

Table 3 shows the total nitrogen absorbed, the amount absorbed from the sodium nitrate added, and the percentage of the latter recovered by the plant.

TABLE 3.—Total nitrogen absorbed by growing trees, and total grams and percentage of nitrogen absorbed from the added sodium nitrate

Treatment	Trees under cultivation			Trees in sod		
	Total N absorbed	N absorbed from added NaNO_3	Added N utilized	Total N absorbed	N absorbed from added NaNO_3	Added N utilized
	Grams	Grams	Percent	Grams	Grams	Percent
Check	53.5	—	—	35.4	—	—
NPK	180.9	127.4	27.4	187.7	152.3	22.5
NP	170.2	116.7	25.1	180.3	144.9	31.2
NK	131.7	78.2	16.8	127.3	91.9	19.5
N	121.3	67.8	14.6	119.0	83.6	18.0

The utilization and recovery of added nitrogen follows the descending series $\text{NPK} > \text{NP} > \text{NK} > \text{N}$. The addition of monocalcium phosphate and potassium sulphate to the sodium nitrate either singly or together has, therefore, increased the efficiency of the latter. The data also show that the addition of monocalcium phosphate has increased the absorption of soil nitrogen, inasmuch as the increase in the amount of nitrogen absorbed by the trees treated with monocalcium phosphate compared with the check trees is 23.6 and 12.5 g in the cultivation and sod systems, respectively. These results may be compared with those of the Rothamsted wheat plots, in which the absorption of nitrogen by the crops on plots treated with potash and phosphates is very much greater than on the plots which received no such additions (22).

RECOVERY OF PHOSPHORUS

In high calcareous soils the addition of monocalcium phosphate results in the formation of insoluble di- and tri-calcium phosphates, the formation of the latter being greater the greater the $\text{Ca}:\text{P}_2\text{O}_5$ ratio. In the presence of magnesium carbonate, similarly difficultly soluble, calcium and magnesium phosphates are formed. Gerlach's experiments (4) may be given as an illustration of the high fixing capacities of calcium carbonate, magnesium carbonate, aluminum hydroxide, and ferric hydroxide for the phosphate ion. The phosphorus held by calcium carbonate and magnesium carbonate, however, is readily dissolved by a saturated solution of carbonic acid, whereas none of the phosphate fixed by the sesquioxides was dissolved.

In the absence of calcium carbonate, superphosphate is supposed to react with iron and aluminum compounds to form basic dialcic aluminum and iron phosphates. This fact explains why the soil solution and the sap of plants contain so little phosphorus following

fertilization with phosphates. The concentration of phosphorus in the soil solution ranges between 1 and 2 parts per million. The drainage water of soils contains only a trace of phosphorus even in sandy soils (6).

The total amount of $\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ supplied to each of the trees that received phosphorus was 1,869.0 g, equivalent to 1,052.5 g phosphorus, and corresponding to an annual application of 350.8 g or 0.77 pounds of phosphorus per tree. The current practice in the experimental orchards of this station is to apply 250 pounds of 16 percent superphosphate per acre of 100 trees, corresponding to 0.45 pounds of phosphorus per tree.

The total amount of phosphorus absorbed, the approximate amount of added phosphorus absorbed from the added phosphate, and the percentage utilized (recovered) is given in table 4.

TABLE 4.—Total phosphorus absorbed by growing trees, and total and percentage of phosphorus absorbed from the added monocalcium phosphate

Treatment	Trees under cultivation			Trees in sod		
	Total P_2O_5 absorbed	P_2O_5 absorbed from added $\text{CaH}_4(\text{PO}_4)_2$	Added P_2O_5 utilized	Total P_2O_5 absorbed	P_2O_5 absorbed from added $\text{CaH}_4(\text{PO}_4)_2$	Added P_2O_5 utilized
	Grams	Grams	Percent	Grams	Grams	Percent
Check	17.6	—	—	12.8	—	—
NPK	31.6	13.9	1.32	32.0	19.2	1.85
NP	27.7	10.1	.95	29.9	17.1	1.62
PK	15.8	—1.8	—	17.3	4.5	.42
P	20.3	2.6	.25	14.0	1.2	.12

The order of utilization and recovery of phosphorus in relation to treatment is $\text{NPK} > \text{NP} > \text{PK} > \text{P}$ except in the PK treated trees under cultivation, in which a negative absorption of added phosphate occurred. The utilization of phosphorus and the recovery of added phosphates by all the trees are very low, the range being from 0.12 to 1.85 percent. This may be compared with the 6.0 percent average recovery of phosphorus from superphosphates in the Rothamsted experiments from field crops (21).

Table 5 shows that the total quantity of phosphorus in the soil used in this experiment is quite small. The sesquioxides are high (aluminum oxide and ferric oxide equal 13.94 percent in the surface soil and 19.16 percent in the subsurface soil), as is also the calcium oxide.

TABLE 5.—Composition (percent) of the soil with respect to its principal basic and acidic constituents

Constituent	Surface soil to 9.5 inches	Subsurface soil, 9.5 to 18.5 inches	Subsoil, 18.5 to 42 inches	Constituent	Surface soil to 9.5 inches	Subsurface soil, 9.5 to 18.5 inches	Subsoil, 18.5 to 42 inches
SiO_2	72.330	67.670	63.115	CaO	0.680	0.655	0.905
Al_2O_3	10.257	13.585	15.984	MgO	.665	1.015	1.360
Fe_2O_3	3.690	5.580	6.840	K_2O	3.925	4.200	4.410
P_2O_5	.098	.100	.077	Na_2O	2.260	1.290	1.315

These high values for aluminum and iron sesquioxides may account for the exceptionally small recovery of phosphorus by the plants in this experiment. It should, however, be emphasized that the mecha-

nism by which phosphorus is rendered immobile in the soil has not been definitely established, and no advances have been made since the writer reviewed the subject (31).

RECOVERY OF POTASSIUM

The total amount of potassium sulphate applied to each of the potash-treated trees was 1,027 g, equivalent to 548.4 g of potash. This corresponds to an annual application of 182.8 g, or to 0.40 pound per tree. Table 6 shows the total amount of potash absorbed, the approximate amount of potash absorbed from the potassium sulphate added, and the percentage of the latter recovered by the plant.

TABLE 6.—Total potash absorbed by growing trees, total and percentage of potash absorbed from the added potassium sulphate

Treatment	Trees under cultivation			Trees in soil		
	Total K ₂ O absorbed	K ₂ O absorbed from added K ₂ SO ₄	Added K ₂ O utilized	Total K ₂ O absorbed	K ₂ O absorbed from added K ₂ SO ₄	Added K ₂ O utilized
	Grams	Grams	Percent	Grams	Grams	Percent
Check.....	65.4			52.1		
NPK.....	140.3	71.8	13.5	136.8	47.2	8.6
NK.....	114.4	49.0	8.9	113.3	61.2	11.1
PK.....	79.7	14.1	2.3	71.1	19.0	3.4

The order of utilization by the trees of added potash follows the descending series NPK > NK > PK. The amount of added potash recovered is relatively low. The range is from 2.3–13.5 percent. In field crops the average recovery of added potassium is 50 percent. The marked influence of sodium nitrate in combination with mono-calcium phosphate and also of sodium nitrate applied singly in increasing the absorption of potassium is apparent. This effect may be attributed to the influence of the sodium and calcium ions of the added salts on the replaceable bases and also to other factors discussed by the writer (31).

RELATIVE PROPORTIONS IN WHICH NITROGEN, PHOSPHORUS, AND POTASSIUM HAVE BEEN UTILIZED

The ratios of N:P₂O₅:K₂O calculated on the basis of phosphoric acid as the unit, are shown in table 7.

In Thompson's experiments (33) the ratio for trees which had the same approximate dry-weight production but which apparently received no fertilizers is 3.9:1:3.4.

An unsolved problem of great practical significance to agronomists and horticulturists is the question as to whether each crop has its specific N:P₂O₅:K₂O ratio requirement, assuming that soil and climatic factors are constant. The results in table 7 lead to the conclusion that the ratios in which these elements are absorbed diverge from one another to a relatively small degree when plants that are optimum with respect to growth and reproduction are compared. In the present experiments, these are the trees which received the NPK and NP treatments.

TABLE 7.—Variation in the ratio of nitrogen, phosphorus, and potash in the trees from cultivated and uncultivated soil to which different mineral treatments were applied

Treatment	EXCLUDING LEAVES			INCLUDING LEAVES		
	Soil under cultivation			Soil in sod		
	N	P ₂ O ₅	K ₂ O	N	P ₂ O ₅	K ₂ O
Check.....	2.37	1	2.68	1.76	1	2.69
NPK.....	5.21	1	3.09	5.37	1	3.06
NP.....	5.59	1	3.08	5.49	1	2.59
NK.....	5.20	1	3.72	4.85	1	3.85
PK.....	3.05	1	3.18	2.13	1	2.51
N.....	5.11	1	3.31	5.11	1	3.41
P.....	3.11	1	2.93	2.72	1	2.57

These results serve to explain the apparently anomalous behavior of the trees which received no potassium, referred to on page 572, in which a relative decrease in the absorption of nitrogen and phosphorus resulted in increased growth under both sod and tillage and in increased blossoming under cultivation. It would appear that there is a very delicate balance between the ratio of these dominant nutrient elements for optimum growth and that the concentration of potassium is too high relative to the nitrogen and phosphorus in the trees which received potassium additions. The potassium concentration exerts an enormous influence in determining the rate of diffusion of carbon dioxide into the cell and up to the chloroplast surface.

COMPARISON OF THE RATIOS OF N:P₂O₅:K₂O ABSORBED BY TREES OPTIMUM WITH RESPECT TO GROWTH AND REPRODUCTION, WITH THE RATIOS IN WHICH THESE ELEMENTS HAVE BEEN APPLIED IN THE FERTILIZER ADDED

The method adopted in ascertaining the amounts of the respective elements which the trees under examination have absorbed from the respective salts added as fertilizer is not a mathematically rigorous one. This is a consequence of variation in the effect factors discussed on pages 570 and 572. The method of calculation is, however, sufficiently exact for all practical purposes. The very wide discrepancy between the ratio in which these elements are absorbed by the trees showing optimum physiological balance and the ratio in which the nutrient salts were applied is clearly brought out. The discrepancy is in part due to the soil properties, viz, leaching of nitrates and fixation of phosphorus and potassium.

After quantitative nature of these effects has been determined by an examination of the soil before and after the experiment, the theoretical economic quantities and ratios for optimum growth and reproduction of trees of this age will be given.

The total amounts of nitrogen, phosphoric acid, and potash added per tree were 464.5:1,052.5:548.4, equivalent approximately to a 3:8:4 fertilizer. In the trees analyzed the optimum ratio in which nitrogen, phosphoric acid, and potash have been absorbed is 175.6:29.7:124.8. By deducting from these values the amounts of nitrogen, phosphoric acid, and potash absorbed by the check trees, we get 122.1:12.1:59.5, or approximately 3.0:0.3:1.5 as the ratio in which these elements have been absorbed from the salts added compared with a 3:8:4 ratio actually applied.

An effort has been made and is still being made to remove fertilizer practice from the realm of empiricism. These results show the difficulties that must be overcome. The analysis of the plant alone is evidently insufficient. A knowledge of the fixing capacity of the soil is obviously of great importance. It follows that the application of the time-absorption-graph method on the entire plant (31) to the determination of the fertilizer requirements of the species when grown on soils of high fixing power necessitates a knowledge of the fixing capacity.

Although Wolff (36) provided definite proof that the composition of the ash of plants varied with the amounts and relative proportion of salts present in the nutrient culture or added to the soil, the present as well as the earlier results (32) cannot fail to emphasize anew the enormous differences that may be produced with respect to the percentage and absolute amounts as well as in the ratio of protein, carbohydrates, and ash constituents as the result of different fertilizer practices.

PRODUCTION OF DRY MATTER PER UNIT OF NITROGEN, PHOSPHORIC ACID, AND POTASH ABSORBED BY THE PLANT

Table 8 shows the amount of growth expressed in terms of dry weight of material formed per gram of the nutrient elements (as N, P_2O_5 , and K_2O) absorbed by the trees in each treatment. The data exhibit another form of the operation of the law of diminishing returns inasmuch as the dry weights produced per unit of nutrient absorbed in the various treatments decrease as the total amount of that nutrient absorbed increases.

TABLE 10.—Quantity of dry matter (grams) produced by the absorption of 1 gram of nutrient from cultivated and uncultivated soils to which different mineral treatments were applied

Treatment	Grams of dry matter produced per gram of indicated nutrient element applied to soil					
	N		P_2O_5		K_2O	
	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod	Soil under cultivation	Soil in sod
Check.....	276	323	840	888	226	219
NPK.....	101	100	584	589	130	138
NP.....	113	105	694	634	176	190
NK.....	134	145	780	785	155	162
PK.....	186	200	745	637	148	155
N.....	127	135	773	781	170	173
P.....	194	207	733	706	187	18

SUMMARY AND CONCLUSIONS

In May 1922, Stayman Winesap apple trees propagated by whip-grafting scions from a single parent tree on roots vegetatively propagated from a single tree were planted in a Hagerstown clay loam soil contained in 5- by 5.5-foot cylinders. Each year from 1925 to 1927, inclusive, applications were made of the pure salts sodium nitrate, monocalcium phosphate, and potassium sulphate in different combinations. The annual application per tree was equivalent to 0.33 pound of nitrogen, 0.77 pound of phosphoric acid, and 0.40 pound of potash. With respect to cultural systems half the trees were in tillage and half in sod.

In October 1927, an entire tree representative of each nutrient treatment and also of each cultural system was removed, and the amounts of nitrogen, phosphorus, and potassium assimilated during the growth period of 6 years were determined.

The percentage and absolute (total) amounts of each of the elements absorbed by the trees during the whole period of growth and also the ratio in which they were absorbed varied greatly with the nutrient treatments and also, but to a much lesser extent, with the cultural system.

The values for the total amounts of nitrogen, phosphorus, and potassium absorbed by an entire tree in each of the respective treatments indicate that the omission of any one of these nutrient elements from the complete fertilizer (NPK) is followed by a decreased absorption of the remaining elements. This decreased absorption resulted in a nutritional lack of balance as exhibited in reduced growth and flowering except in one case in which potassium was omitted.

The Wirkungswert (the effect factor) for nitrogen, phosphorus, and potassium, varied with the elements with which the element considered was associated in the added fertilizers. These effect factors are discussed in detail.

The percentage recovery by the trees of added nitrogen, phosphorus, and potassium is relatively low under all treatments.

There appears to exist a specific ratio in which nitrogen, phosphorus, and potassium are absorbed by the trees (NPK and NP) which are optimum with respect to growth and reproduction. This ratio is approximately 6:1:4.

The wide discrepancy shown between the ratio in which these principal nutrient elements are absorbed by the trees—even by those showing optimum physiological balance (NPK and NP)—and the ratio in which the nutrient salts were applied is indicative of soil effects, namely, leaching of nitrates, and of high fixing capacities of phosphorus and potassium.

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Oat Hulls as a Source of Vitamins B and G*

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OAT hulls, as an ingredient of mixed feeds for livestock, have been regarded with disfavor by many feeding authorities and, as a result, many states have enacted laws regulating the sales of mixed feeds, which limit the amount of crude fiber that may be added in the form of oat hulls. This viewpoint has developed as a result of routine chemical studies which show that oat hulls consist largely of crude fiber, which is considered a non-essential ingredient and which adds but little to the nutritive value of the ration other than to furnish "roughage" or "bulk."

In their studies of hock disease in poultry, Hunter, Dutcher, and Knandel (1931) have found that the addition of oat hulls to the hock disease-producing ration will prevent the onset of the abnormality. This and other work of the biological response type has suggested that oat hulls may possess nutritive properties which cannot be predicted on the basis of chemical analysis.

Previous observations led us to believe that certain beneficial effects obtained by feeding oat hulls to rats could not be explained on the basis of its fibrous nature or its protein or mineral content. In fact, preliminary experiments had shown that our basal ration, deficient in the vitamin B-complex, could be supplemented to better advantage with oat hulls (20 percent) than with an equal weight of ground wheat. This

observation led to the belief that the vitamin B-complex content of oat hulls could be studied with profit. The present paper represents the results of a study of the relative amounts of vitamins B and G present in a typical commercial sample of oat hulls.

EXPERIMENTAL

The oat hulls used in this investigation were free from groats, cracked grain, and dust. After drying, the oat hulls were ground in a Wiley mill and stored for future use. The vitamin-B supplement used in this study was prepared by percolating 95 percent alcohol through brewers' yeast as long as the percolate remained colored. This percolate was evaporated under diminished pressure to a semi-solid consistency and the residue was dried in vacuum over sulfuric acid, after which it was macerated with 95 percent alcohol, filtered and the filtrate made up to such a volume that 0.1 ml. represented one gram of brewers' yeast.

The vitamin-G supplement was prepared from bakers' yeast by adding sufficient 95 percent alcohol to a filtered aqueous extract of the yeast to bring the alcoholic content of the mixture to 50 percent by volume. The resultant precipitate was filtered off and discarded. Additional alcohol was added until the concentration reached 80 percent by volume. The precipitate, which formed at this alcoholic concentration, was removed by filtration, dried at room temperature, moistened with distilled water, and autoclaved for six hours at 15 pounds pressure. It was then dried, pulverized, and sufficient

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finely ground dextrin was added to make 0.3 grams of the resultant mixture equivalent to 1 gram of the original yeast.

The basal diet used throughout the investigation consisted (in parts per 100) of washed casein 18, salt mixture (McCollum's 185, 1918) 4, agar 2, sucrose 15, cod liver oil 2, ~~and~~ filtered butterfat 3. The cod liver oil and butterfat were added to

and dextrin 56.

TABLE 1. Groups of animals used and their respective experimental diets

Group	Basal ration (parts per 100)	Oat hulls (parts per 100)	Vitamin B supplement (mls.)	Vitamin G supplement (gms.)	Average weekly feed intake during experi- mental period
1	100	16
2	100	0.3	21
3	100	...	0.1	...	20
4	95	5	24
5	90	10	32
6	80	20	36
7	80	20	0.1	...	40
8	80	20	...	0.3	41
9	100	...	0.1	0.3	33

All animals were continued on the experiment for at least eight weeks after the depletion period, unless death intervened. The results obtained through this series of experiments are shown graphically in Figure 1. The curves represent the average growth responses of all animals in the respective groups.

the other constituents of the diet just prior to feeding.

Piebald rats 20 to 21 days old and weighing 39 and 45 grams each were placed in individual cages, provided with raised screen grids, and fed liberal quantities of the basal ration. The bottoms of the cage pans were covered with sheets of absorbent paper, which were changed at least once each week. Soiled screens were replaced when necessary by clean ones. A weekly record was made of the quantity of food consumed, the changes in weight, and the general appearance of each animal. The animals were maintained on the basal diet until cessation of growth occurred. This usually required from 10 to 24 days. At this time they were arranged in groups of from 6 to 12 animals each, special care being taken to distribute sexes and litters throughout the several groups. It should be pointed out that, in ar-

ranging the experimental groups, all animals were maintained in individual cages throughout the experiment and liberal amounts of the basal ration were supplied at all times. The nine groups of animals which were used in this phase of the investigation and their dietary regimen are given in Table 1.

In addition to the above, a second series

of experiments was conducted in which the oat hulls were partially fractionated and each of the fractions were fed to groups of animals as the sole supplement to the basal diet. The method of fractionation was as follows: A weighed quantity of the ground oat hulls was extracted with 95 percent alcohol until no further color was removed. This extract was concentrated under diminished pressure to a small volume and diluted with alcohol until 1 ml. of the extract was equivalent to 10 grams of the original oat hulls. After the extracted hulls had been practically freed from alcohol, the hulls were placed in a flask and sufficient distilled water was added to cover completely the fibrous mass. The flask and contents were then heated on a steam bath for one hour, and the aqueous extract was removed by pouring the contents into a large Büchner funnel and filtering by means of suction.

The hulls were returned to the flask, distilled water was again added, and the above process was repeated two additional times, making a total of three aqueous extractions. The combined aqueous extracts were evaporated to dryness, and the residue was pulverized and weighed. One gram of the resulting powder represented 20.8 grams of the original hulls. The extracted hulls were also dried and weighed.

Four groups of rats (6 animals per group) similar to those previously described, were used in this phase of the investigation. The groups and their dietary treatments were:

Group 1 received a diet composed of 80 parts of the basal diet and 20 parts of the ground oat hulls.

Group 2 received a diet composed of 80 parts of the basal diet and 20 parts of the extracted hulls.

Group 3 received the basal diet supplemented by the alcoholic extract equivalent to 20 percent of the hulls.

Group 4 received the basal diet and, in addition, the aqueous extract equivalent to 20 parts of the hulls for each 100 grams of food.

These animals were continued on experiment for at least eight weeks after the depletion period unless death intervened. The growth responses made by these groups are shown in Figure 2. Since males and females were equally distributed in the several groups, each curve represents the average growth of all animals comprising the group. The average weekly food intakes for each of the four groups, during the eight-week period, were 35, 16, 19, and 17 grams.

All animals from the three last-mentioned groups (Groups 2, 3, and 4) surviving at the end of the eight-week experimental period were placed on a diet composed of 80 parts of the basal diet, and a recombination of the extracted hulls, the alcoholic extract, and the aqueous extract, equivalent to that obtained from 20 parts of the

original hulls. These animals remained on this diet for three additional weeks before the experiment was terminated. The growth responses as a result of these changes in diets are represented by the broken line extensions of the growth curves (Fig. 2).

DISCUSSION

It has been recognized for some time that rolled oats, like other cereals, is a satisfactory source of vitamin B, but this cereal has been considered a questionable source of vitamin G. After considering the available information concerning the cereal grains, our results were somewhat surprising, i.e., that oat hulls contained as much vitamin G as vitamin B, and with some evidence that the former is present in slightly greater quantities (relatively) than the latter.

When the basal diet was supplemented by 5 percent of the hulls, there was a small but definite response in growth which lasted for three weeks (Curve 4, Fig. 1). After this time the average weight of the animals gradually declined, and a number of animals developed symptoms of beriberi. When the supplement constituted 10 percent of the diet, the animals showed a slow but consistent gain in weight throughout the experimental period (Curve 5, Fig. 1). When the oat hulls were included in the diet to the extent of 20 percent, the animals grew at a very uniform rate, which resulted in an average weekly gain of 5 grams per week for the eight-week period (Curve 6, Fig. 1).

The vitamin B and G concentrates, when used as supplements to the basal diet, in addition to 20 percent of oat hulls, showed some beneficial effects upon growth (Curves 7 and 8, Fig. 1). It appears that the vitamin B concentrate was slightly superior as a supplement to such a diet.

We wish to call attention to the fact that the protein levels varied to some extent owing to the dilution of the basal ration with protein poor oat hulls. At no time, how-

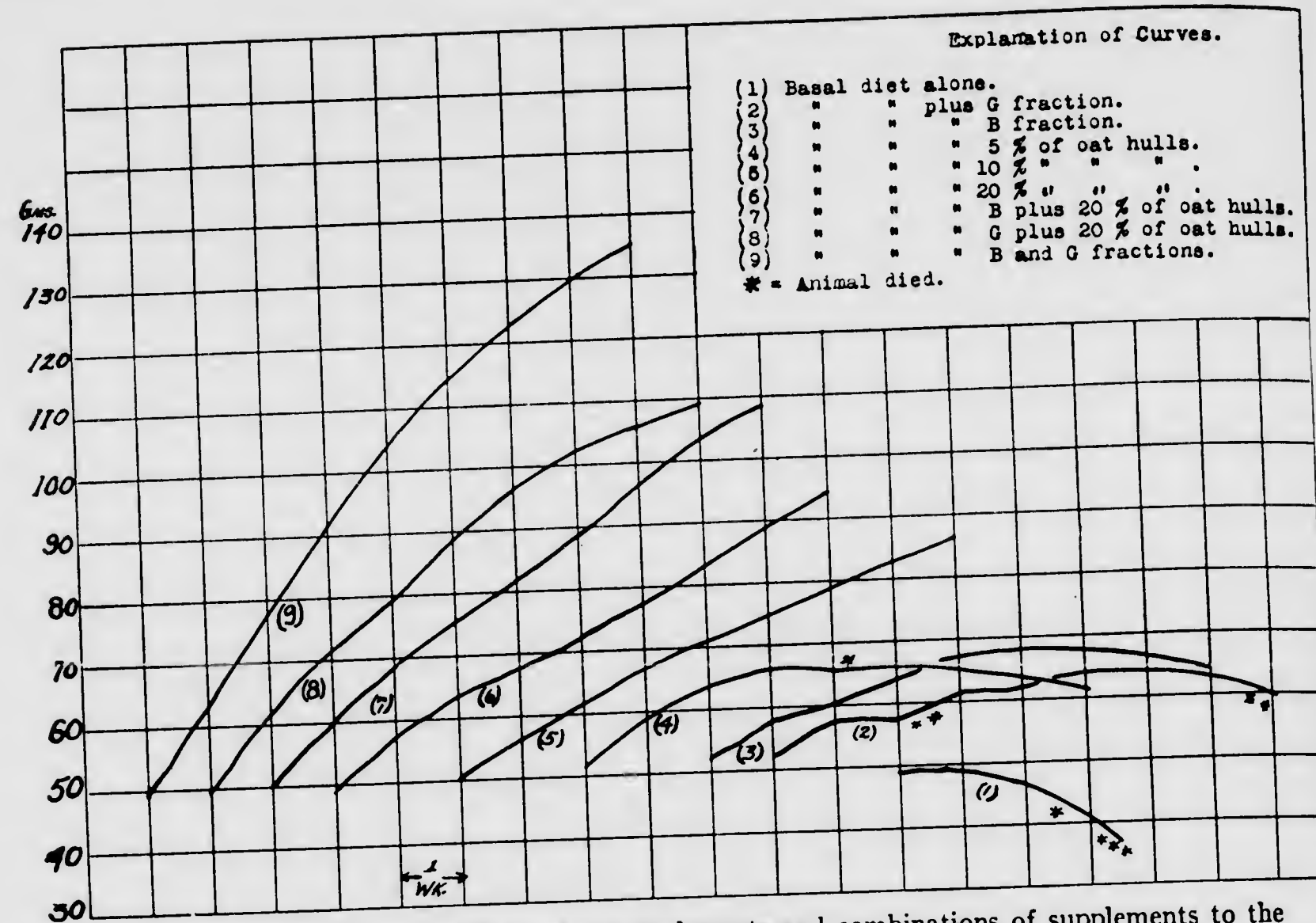


FIG. 1. Showing the effect of the various supplements and combinations of supplements to the basal diet on growth.

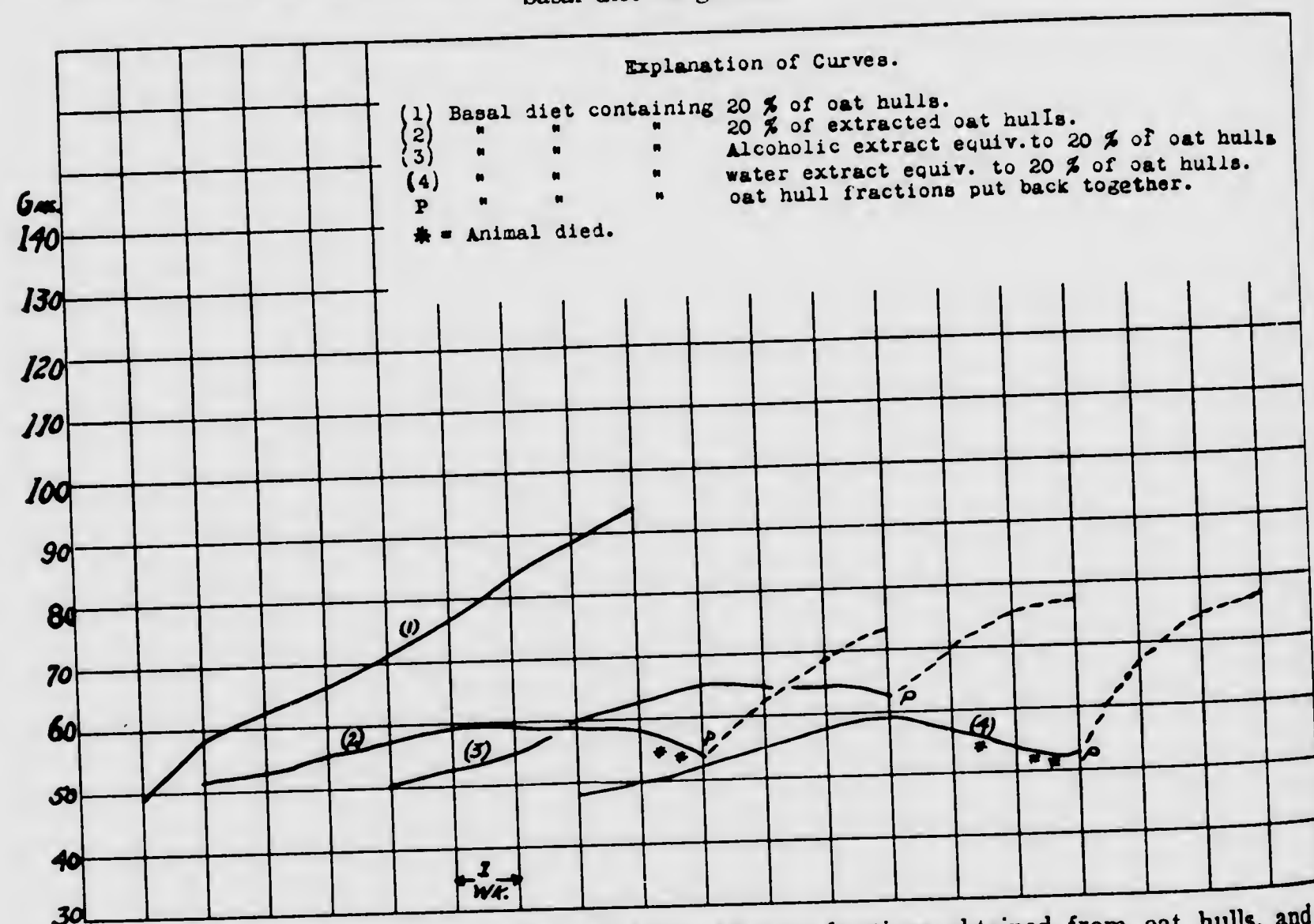


FIG. 2. Showing the supplementary effects of the different fractions obtained from oat hulls, and the results of a recombination of these fractions.

ever, was this variation greater than 3 percent. If this variation is taken into consideration, our data indicate that the supplementing effect of the oat hulls might have been more pronounced had the protein content of the diet been kept constant.

It will be noted that while the average feed intakes of the animals of the control group (Curve 1, Fig. 1), and the extracted oat hulls group (Curve 2, Fig. 2) were practically equal, the animals of the latter group lived longer. This was believed to be due not to traces of the vitamins left in the extracted residue, but rather to a favorable effect of the fibrous residue. Whether this effect is due to a more favorable condition for enzymatic and bacterial action in the digestive tract, resulting in slight vitamin synthesis by the bacteria or to a third and less soluble fraction of the vitamin B-complex, we are not in a position to state at the present time.

SUMMARY

1. Oat hulls were tested for their vitamin B and G content and were found to contain

an appreciable quantity of each of these factors.

2. The life of animals receiving the basal diet supplemented by 5 percent of oat hulls was greatly extended by this supplement.

3. Ten percent of oat hulls resulted in slow but consistent growth while 20 percent of hulls resulted in a five gram gain per week during the eight-week period.

4. Vitamin B and G concentrates appeared to be about equally effective in stimulating additional growth.

5. While 20 percent of extracted hulls as a supplement to the basal diet was not sufficient to stimulate growth, there were some evidences that it did prolong life.

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CODLING MOTH IN PENNSYLVANIA*

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The distribution of codling moth (*Carpocapsa pomonella*) is statewide. The topography of Pennsylvania has a modifying effect on the nature of the infestations which renders the insect more virulent in the southcentral counties. In the higher levels where the season develops later the injuries are always lighter. In no section are apples immune and orchards poorly sprayed usually rank high in codling moth damage.

The broods of codling moth vary somewhat. There appears to be but a single complete brood and a small second brood of worms through most of the area north and west of a diagonal drawn from the northeastern corner to a mid-point on the southern boundary of the state. On a relief map this will coincide very closely with the coastal plain levels although it contains the Blue Mountain ridges. Local areas of heavy infestation occur on the higher levels and along the main water courses, notably in Erie County, Allegheny County, the lower Juniata Valley and the upper Susquehanna River to the confluence of its branches at Sunbury, Northumberland County. Throughout the higher levels in the Allegheny Mountains and west of State College the codling moth is a constant threat but control is fairly easy. In the upper half of the eastern coastal region there is a full first brood with a small second brood, and in the central and lower eastern half a full first brood and larger second. But in the lower reaches of the Susquehanna River and in the Cumberland Valley this second brood is still more nearly complete, and in some years a partial third brood has been suspected. Some of the difficulties with which we have to contend may be shown in a graph representing emergence and length of the periods of moth infestation. Attention is called to the variation in emergence of the wintering brood over the period 1925-1933. Also to the length of flight periods as indicated by the graph (Figure 5).

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fruit cover sprays and weather conditions is given in Table 2. For convenience in comparison the tolerance used is .01 grains of arsenic per pound of fruit, irrespective of earlier tolerance specifications. This year only seven samples showed arsenic trioxide slightly above .005 grains per pound of apples and of these two were .006; one, .007; two, .008; and two, .009 grains.

TABLE 2. ANALYSES OF APPLES FOR RESIDUAL ARSENIC 1926-1933

Year	No. of samples	Cover sprays	Seasonal dosage	Weather conditions	Residue conditions		
					Tolerance		
		No.	Lbs.		Above No.	Equal No.	Below No.
1926.....	24	3	9	excess rain	0	0	24
1926.....	12	4	12		0	0	12
1930.....	28	3	9	dry	7	0	21
1930.....	68	4	12		14	8	46
1931.....	15	3	9	dry	3	0	12
1931.....	65	4	12		8	2	55
1932.....	47	3	9	mod. dry	2	0	45
1932.....	88	4	12		8	0	80
1933.....	6	3	9	excess rain	0	0	6
1933.....	6	4	12		0	0	6
1933.....	44	3	7		0	0	44
1933.....	13	4	9		0	0	13

In 25 additional orchards in this year's series where two cover sprays were applied, using a dosage of 2½ pounds of lead arsenate and 2 pounds of powdered milk to each 100 gallons, the arsenic trioxide per pound of apples ranged between a trace and .002 grains per pound of apples. Three cover sprays were put on in seven orchards using either calcium arsenate at the rate of 3 pounds to each 100 gallons of dilute spray, or lead arsenate 2½ pounds plus calcium arsenate 3 pounds to each 100 gallons of spray. The combinations of lead arsenate and calcium arsenate were either two pounds of lead and one pound of calcium arsenate or one of calcium arsenate and two of lead arsenate. No appreciable differences were noted in the arsenic trioxide where the combinations were used and it was either a trace or .001 grains per pound of apples. The apples treated with calcium arsenate at the rate of three pounds to each 100 gallons of dilute spray showed only .002 grains of arsenic trioxide per pound of apples. Some 21 samples were taken from orchards where control was poor due to practices used and on these apples not more than .001 grain of arsenic trioxide was found.

The lead residues were analyzed this year according to a modification of the method of Lynch,² as used by Percival and Potter.³ For

²Lynch, W. D., et al. Poisonous Metals on Sprayed Fruits and Vegetables. U. S. D. A. Bul. 1027, 1922. Percival, G. P. and Potter, G. F.

³Amount and Variability of Spray Residue on New Hampshire Baldwins. N. H. Tech. Bul. 49, 1932.

this purpose 58 samples were selected from the lots collected and these represent the average conditions in completely sprayed commercial orchards. Four of these samples when analyzed had a lead content in the residue above the tolerance of .02 grains of lead per pound of apples. The highest amount found was .03 grains per pound of fruits and the most of them were between .004 and .009. Only five of them ranged between .02 and .03 grains per pound of apples. There was no relation between the amounts of arsenic and the amounts of lead in the same sample. The larger amounts of lead were, however, from samples taken in either our demonstration orchard in Franklin County or in orchards where the fruit growers spray their trees according to the method we have demonstrated to them and use in our own operations.

EXPERIMENTAL DATA ON CODLING MOTH CONTROL.—The foregoing discussion is of general orchard conditions with especial reference to the effectiveness of the control program suggested through the Agricultural Extension Service.

A codling moth research program was initiated in 1928, but until 1931 the work was limited to a study of timing methods for spray applications⁴ and the use of supplementary control methods, notably banding.⁵ Bait pails appear to be more satisfactory than emergence cages in heavily infested orchards, because they indicate more clearly the periods of moth flight. During the past two years they have been used successfully in a number of commercial orchards. The scraping and banding of apple trees is rapidly becoming a general practice where the codling moth is a problem.

In 1931 spraying experiments conducted in a heavily infested Adams County orchard showed the necessity for four first brood cover sprays and the advisability of additional second brood sprays in such orchards. No undue residue of arsenic resulted from four cover sprays ending July 1. In 1932 the application of five cover sprays ending in mid-July reduced the codling moth population by half, but produced a residue of .028 grains of As_2O_3 per pound at picking time. Comparisons of spray combinations in 1932 indicated that the addition of casein or miscible oil would allow no reduction in the dosage of lead arsenate. A complete cover spray schedule of nicotine tannate with flotation sulphur was unpromising. However, fish oil at one quart per 100 gallons of spray increased the effectiveness of the standard spray mixture. It was not used in 1933 for two reasons. First, its nauseating odor when mixed with lime sulphur would force the use of a substitute

⁴Pa. Agr. Exp. Sta. Bul. 277, 1932.

⁵Jour. Ec. Ent. Vol. 25, No. 6, pp. 1133-1143, 1932.

fungicide not recommended by our pathologists. Second, it was feared that its marked adhesive properties would lead to danger of excessive residues of arsenic and lead, even if it were used in only one spray application.

The basis for this fear was a study of arsenical residues conducted at State College in 1932, which showed that both the arsenic and the degree of control were reduced by the addition of hydrated lime and increased by fish oil. Fruit given three cover sprays with hydrated lime fell under the tolerance, while two cover sprays containing fish oil fell above. In Table 3 are presented figures from a plot receiving lead arsenate at 3-100 in five cover sprays to show the effect of spray applications, growth of fruit, and weather conditions upon the amount of arsenic per unit of fruit surface. This treatment left .021 grains of arsenic trioxide per pound on the picked fruit.

TABLE 3. LOSS OF ARSENIC FROM NEW YORK APPLES AT STATE COLLEGE, PA., 1932*

	June 10* to June 20	June 21* to July 5	July 6* to July 24	July 25* to Aug. 4	Aug. 5* to Oct. 19
Ave. daily rainfall— <i>inches</i>	0.151	0.140	0.150	0.264	0.988
<i>Surface area of fruit</i>					
Per cent of total	8-17	17-28	28-44	44-54	54-100
Times increase during period	2.2	1.7	1.5	1.2	1.8
<i>As₂O₃—gms. per square meter of fruit surface</i>					
After spraying	1.058	1.275	1.034	1.148	1.590
Lost during period	0.686	1.072	0.770	0.548	1.467
Expected loss from growth	0.577	0.525	0.344	0.191	0.883
Per cent loss due to growth	84.1	49.0	44.7	34.8	60.2

*Spraying dates.

The "expected loss from growth" has been computed by dividing the amount of arsenic after spraying by the "times increase" in fruit surface during the period of loss, which gives the amount expected to remain if growth were the only factor concerned. The table brings out the following points.

1. During June, when the fruit was increasing rapidly in size, loss of arsenic per unit of surface was correspondingly high. The need of

*From unpublished thesis of W. S. Hodgkiss, Dept. of Agri. and Biol. Chemistry, The Pennsylvania State College.

accurate timing of spray applications to meet the peaks of egg hatching during this period was indicated even with intervals only 10 to 15 days between sprays.

2. If spraying stopped on June 21, when the fruit was only 17 per cent of its final size, subsequent growth would reduce the amount per unit area on the picked fruit to one-sixth the amount at the date of application. This fact was pointed out by Hamilton⁷ whose studies indicated that the early cover sprays are not dangerous from the standpoint of arsenic tolerance.

3. As the season progressed and the fruit approached more closely its final size, less of the arsenic per unit area lost between spray applications was due to fruit growth. Thus by July 6 nearly 30 per cent of the fruit surface was present, and a third spray applied on this date produced .008 grains of As₂O₃ per pound at harvest. The table shows that where spraying was continued to August 5, when the fruit was over half grown, the arsenic on the picked fruit reached .021 grains per pound in this dry season, even though fruit growth accounted for 60 per cent of the loss during this period. It appears that in any year each spray application after the latter part of June will depend to a greater extent upon rainfall, which cannot be predicted to bring residues below the established tolerance at picking time. The recommendation of the U. S. Department of Agriculture in the spring of 1933 that lead arsenate be omitted after the second cover spray in our section was well founded, even though our 1933 experience indicated that three cover sprays at standard dosage would have been safe with the heavy August and September rains.

SUMMARIZATION.—Over most of Pennsylvania the small size of the second brood of codling moth larvae allows control to be obtained through a schedule of early sprays, without danger of excessive residues of lead or arsenic remaining on the picked fruit.

In the export apple growing region of southcentral Pennsylvania, the development of a large second brood of worms, and of a possible small third brood in favorable seasons, requires lead arsenate at standard dosage in three or four cover sprays extending to the middle or latter part of July. For the most part the necessary schedule has been applied without bringing arsenic above the tolerance.

In orchards where large populations of codling moth have been built up control constitutes a special problem. Often this relates to individual orchards. Four first brood cover sprays, additional second brood sprays,

⁷Jour. Ec. Ent. 22: No. 2, pp. 387-396, 1929.

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and banding of trees are indicated. In the absence of substitutes for arsenic that can be recommended without qualification, our experimental studies indicate that adequate dosage and amounts of lead arsenate produce a danger of excessive residues after the latter part of June, and that fruit washing must be resorted to in heavily infested orchards in comparatively dry seasons.

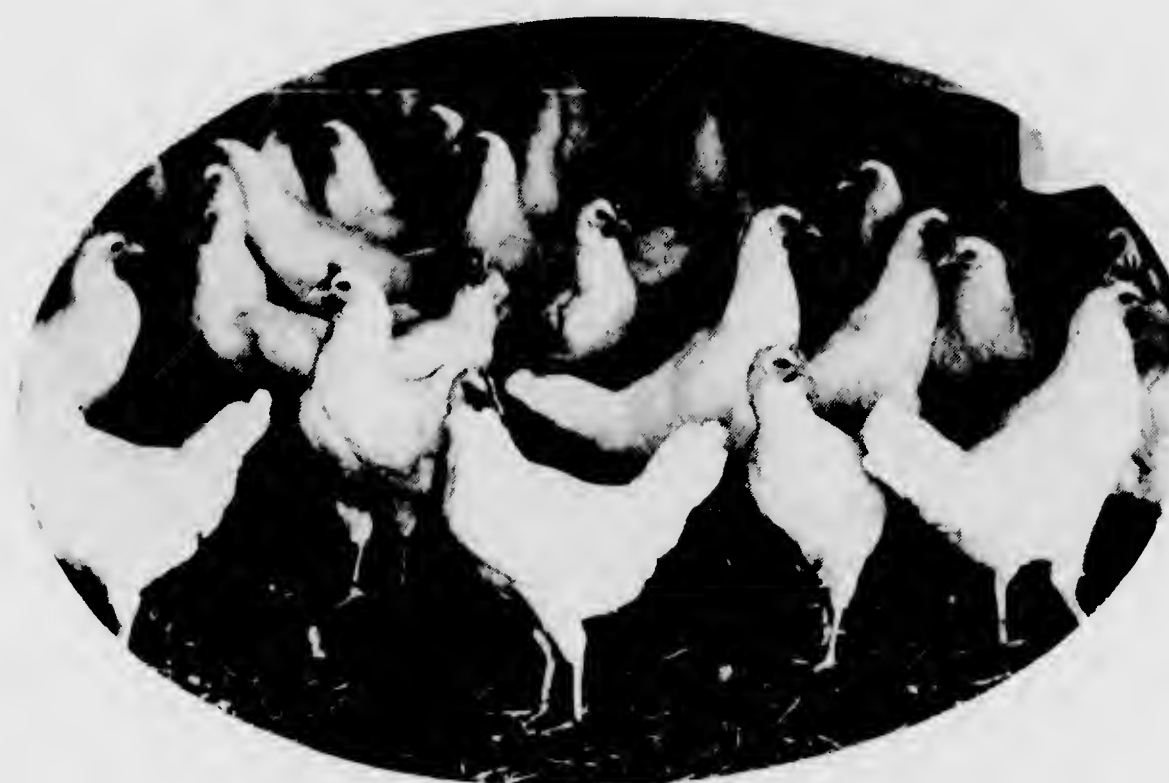
The general orchard survey shows that a rather high amount of commercial control can be obtained under the present standard system without danger from an excess of either arsenic or lead residue. To obtain this efficiency the dosages used must be correct, the applications timely, coverage of leaves and fruits complete, and the spraying equipment must be sufficient to do the job correctly.

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BULLETIN 303

FEBRUARY, 1934

The Vitamin D Requirements of Growing Chicks and Laying Hens



EXPERIMENTAL PULLETS, 16 WEEKS OLD,
WHICH RECEIVED ADEQUATE VITAMIN D

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The Vitamin D Requirements of Growing Chicks and Laying Hens^{1, 2, 3}

R. R. MURPHY, J. E. HUNTER, AND H. C. KNANDEL

WITHIN the last few years, marked changes have occurred in the methods employed in rearing and managing poultry. Chicks are being hatched earlier in the season than was formerly the common practice. Early hatching has made it necessary to confine the chicks for a part of the rearing period; in many instances the fowls are confined from hatching through maturity. This practice has proved advisable when range conditions are unsatisfactory.

Chicks now are being produced and reared throughout the entire year. To make this possible, it is essential that they be supplied with some form of vitamin D. Winter sunshine is not so effective as summer sunshine in the prevention of rickets in chicks. Hart, Halpin, and Steenbock (1922) found that cod liver oil would prevent the appearance of rickets in confined chicks. This work was further substantiated by Mitchell, Kendall, and Card (1923).

Most of the investigations of the requirements of growing chicks for vitamin D as supplied by cod liver oil have related only to the growing period. The work here reported, which covered a period of two years, is a study of the vitamin D requirements of growing chicks and laying and breeding hens. The investigation also included studies on the effect of various levels of cod liver oil fed to the mother hen on the resultant offspring. The cod liver oil used was fortified with vitamin D from cod liver oil and assayed with both chicks and rats to determine its vitamin D potency. Three independent laboratories cooperated in testing this oil. By the rat assay, it was found to contain 100 Steenbock units or 270 International units per gram when the conversion factor 2.7 was used to express the potency in International units. At present, there is no generally accepted recommendation as to the vitamin D requirement of the laying hen. In fact, it has been assumed that the laying hen and the growing chick require similar amounts of vitamin D.

The principal objectives were to determine the effect of varying levels of cod liver oil on (1) growth, bone formation, and general physiological development of growing chicks when fed a well-balanced ration; (2) egg production and the maintenance of body weight in laying pullets; (3) egg weight, amount of egg shell, calcium content of the blood, and the degree of bone calcification; (4) the hatchability of eggs produced by the pullets; and (5) the effect of the vitamin D reserve carried over from the parent to the offspring.

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³ This is a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agricultural and Biological Chemistry in the Graduate School of The Pennsylvania State College.

Experimental Procedure

Two thousand Single Comb White Leghorn chicks of The Pennsylvania State College strain, hatched April 12, 1932, were used. Ten groups of 200 chicks each were selected. Each chick was individually weighed and wing banded at hatching time. These chicks were subjected to the following experimental conditions.

Group number	Environmental conditions	Percent fortified cod liver oil in all-mash ration
1	Battery brooder—confined	None
2	Battery brooder—confined	1/32
3	Battery brooder—confined	1/16
4	Battery brooder—confined	1/8
5	Battery brooder—confined	3/16
6	Battery brooder—confined	1/4
7	Battery brooder—confined	3/8
8	Battery brooder—confined	1/2
9	Colony brooder house—limited range	None
10	Colony brooder house—limited range	1/8

Groups 1 to 8 were brooded in battery brooders, each deck of which was heated indirectly by hot water so that all decks could be maintained at a uniform temperature. The brooders were located in a well-ventilated room equipped with auxiliary heat. No direct rays of the sun were allowed to enter the room.

Groups 9 and 10 were brooded in colony brooder houses of similar size and construction, heated with hard coal burning brooder stoves. The chicks in both groups had access, at all times, to ranges of adequate size that were sown to oats.

All groups were fed the following all-mash rations:

	Basal ration for young chicks (Fed from one-day old to 12 weeks)	Basal ration for growing stock and laying pullets (Fed from 12 weeks to the conclusion of the experiment)
Ground yellow corn	43.5 pounds	45.5 pounds
Wheat bran	10.0 "	10.0 "
Standard wheat middlings	10.0 "	10.0 "
Alfalfa leaf meal	5.0 "	5.0 "
Meat scraps (55% protein)	10.0 "	7.5 "
Dried milk	10.0 "	7.5 "
Ground heavy oats	10.0 "	10.0 "
Ground limestone (98% CaCO ₃)	1.0 "	4.0 "
Salt	0.5 "	0.5 "
Total	100.0 "	100.0 "

The mashes were mixed at the college poultry farm as needed. From chemical analyses it has been found that meat scrap varies considerably, particularly in its mineral content. To overcome this variation, meat scrap was purchased in ton lots and remixed before using. A fresh supply of mash was mixed with fortified cod liver oil at intervals not exceeding two weeks. The oil was weighed in grams and the feed in pounds. Care was taken to have the cod liver oil evenly distributed throughout the mash.

Chemical analyses of the basal mashes made from time to time showed very little difference in composition. The starting mash averaged 18.4 per cent protein, 1.47 per cent calcium, and 0.92 per cent phosphorus. The average analysis of the growing and laying mash was 16.4 per cent protein, 2.79 per cent calcium, and 0.80 per cent phosphorus. Chicks deprived of direct sunshine and fed the basal starting ration showed evidence of rickets when three and one-half weeks old; at 10 weeks of age all chicks in this group showed definite rachitic symptoms.

When four weeks old, the cockerels from all groups were removed from the experiment. The pullets in the eight confined groups were moved from heated to unheated batteries in another room of the same building, in which a uniform temperature was maintained by means of auxiliary heat. The confined groups remained there until moved to the laying pens at 12 weeks of age, when the growing and laying mash was substituted for the starting mash.

Sixty-five representative pullets from each of the 10 groups were selected for the laying pens. Body weight, health, condition, physical development, and state of maturity were considered in the selection. The 10 groups of pullets were housed in a continuous laying house. All pens were of similar construction except the fronts of the two pens in which the range-reared birds were housed. The front of each pen of the eight confined groups had four window sashes hinged at the bottom and swung in at the top for ventilation. Boards were placed at the sides of the sashes to keep out the sunlight. The glass of each sash was kept covered with a soap compound to prohibit any ultraviolet light from entering the pens. The fronts of the pens which housed the two outdoor groups were equipped with a muslin curtain opening, and one double sash glass window to which no soap compound was applied. The pullets of these two groups had access at all times to approximately one-fourth acre of alfalfa range.

Results, Period of Growth, 0 to 24 weeks

Body Weight.—Chicks of all groups were individually weighed at time of hatching and at bi-weekly intervals for 24 weeks. The weights were recorded in grams and the average weight per 100 chicks converted to pounds, (Fig. 1). A more uniform rate of growth was obtained in Groups 9 and 10, reared on range, than in any of the confined groups reared in battery brooders. For the brooding period of 10 weeks, the

physical development, growth, feathering, and pigmentation were very satisfactory for all groups except Group 1 (no cod liver oil) and Group 2 (one-thirty-second per cent cod liver oil).

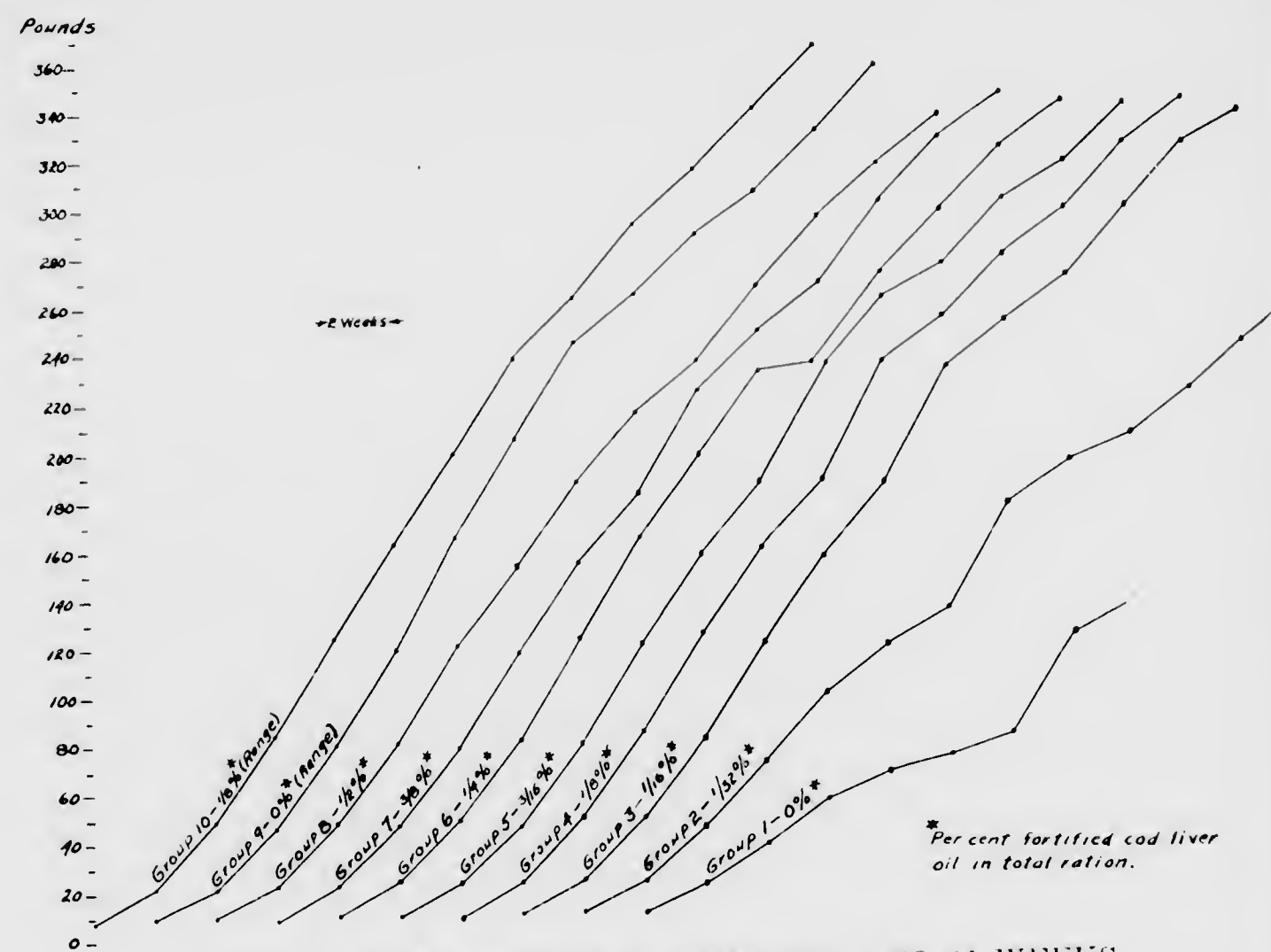


FIG. 1. BODY WEIGHT OF FEMALES, 0 TO 24 WEEKS

(Average weight in pounds per 100 chicks)

Feed Consumption.—With the exception of varying levels of cod liver oil, all groups received the same ration, with no supplements. All feed consumption was recorded at bi-weekly intervals (Table 1). When the pullets were moved to laying quarters the feed consumption decreased markedly from the 12th to the 14th weeks. Several days were required for the birds to become accustomed to the new environment. With the exception of Group 2, all groups consumed approximately the same amount of feed. Group 1 was discontinued at the end of the 16th week because of the extreme rachitic condition of all individuals. All birds in this group exhibited a severe diarrheal condition.

TABLE 1. FEED CONSUMPTION

(Average number of pounds of mash consumed per 100 pullets from 0 to 24 weeks)

Group number	1	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	0	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Weeks										
0-2	33.8	36.3	36.3	36.1	33.2	36.5	35.6	34.1	45.2	47.5
2-4	63.1	71.7	75.8	78.6	70.5	73.3	71.2	76.7	72.9	78.6
4-6	75.7	88.1	113.9	117.6	112.2	113.0	107.1	108.6	117.1	116.0
6-8	85.1	111.6	137.1	137.0	138.8	137.8	132.2	135.9	141.1	151.3
8-10	79.3	129.3	168.1	168.9	171.3	182.2	166.3	162.1	177.5	191.1
10-12	83.9	121.1	173.3	179.0	189.3	187.2	174.9	187.8	214.5	214.7
12-14	53.4	73.9	127.1	126.1	123.1	108.5	107.2	92.9	119.3	121.7
14-16	152.0*	161.9	178.6	191.3	169.8	178.8	193.1	201.1	207.2	171.8
16-18		136.4	209.3	221.0	207.7	235.2	215.1	201.0	216.1	253.5
18-20		175.4	225.4	208.0	229.7	225.8	227.0	227.0	201.5	238.5
20-22		189.3	234.9	217.4	235.1	233.9	240.2	245.0	240.4	245.8
22-24		201.9	266.3	255.7	254.5	251.5	253.2	261.0	243.3	280.5
Total pounds of mash consumed, 0 to 24 weeks	626.2	1502.1	2947.1	3067.0	3075.2	3063.7	3029.0	2902.4	3096.3	2443.0

* Group 1 was discontinued at the end of the 16th week because of the extreme rachitic condition of all individuals.

Vitamin D Intake.—From assays conducted by three separate laboratories, the fortified cod liver oil used throughout this experiment was found to contain 100 Steenbock units per gram. Using the conversion factor 2.7, this lot of fortified cod liver oil was computed to contain 270 International Units of vitamin D per gram. The average number of International Units of vitamin D consumed per female during the first 24 weeks in each of the groups is given in Table 2.

TABLE 2. VITAMIN D INTAKE

(Average number of International Units from cod liver oil consumed per pullet from 0 to 24 weeks)

Group number	1	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	0	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Weeks										
0-8	0	118	275	595	815	1,104	1,603	2,176	0	607
8-16	0	187	496	1,019	1,569	2,011	2,946	3,961	0	1,121
16-24	0	270	716	1,127	2,129	2,898	4,310	6,123	0	1,559
Total number of units, 0 to 24 weeks	0	575	1,490	3,011	4,513	6,012	8,559	12,202	0	3,287

* Discontinued at 16 weeks.

Bone Ash Determinations.—The results obtained from the bone ash and blood calcium determinations made at 8, 16, and 24 weeks are given in Table 3. The percentage of bone ash at 8, 16, and 24 weeks is the average of four tibiae taken from the right leg of four chicks from

each group and ashed separately. Four chicks weighing within a few grams of the average weight for the group were used. The bones were preserved in alcohol. All adhering muscle and connective tissues were removed but the proximal and distal cartilaginous caps were not removed. The bones then were dried in an oven at 105 degrees C. for 24 hours. All bones were split longitudinally, placed in asbestos cones and extracted with hot 95 per cent alcohol for a period of 20 hours in a Soxhlet apparatus, followed by a similar treatment with ether. After extraction, the bones were dried to constant weight and burned in an electric muffle furnace to a white ash. Crucibles containing the bones were placed in a cold furnace and the temperature gradually increased until ashing was complete.

Considerable variation has been found in the percentage of bone ash of chicks similarly treated. The percentages of bone ash obtained in this work from the tibiae of 8-week-old chicks (Table 3) agree closely with those reported by Bethke and associates (1929). Using the tibiae with those reported by Bethke, these workers obtained bone ash values from normal 8-week-old chicks, these workers obtained bone ash values of approximately 52 per cent. For groups in which all chicks were rachitic at eight weeks of age the values they obtained varied from 33 to 42 per cent. Both alcohol and ether were used for extraction. The values found in our work are somewhat higher than those reported by Wilgus (1931) who found bone ash values averaging approximately 45 per cent for normal 8-week-old chicks. For mildly rachitic 8-week-old chicks, he reports an average approximating 40 per cent. Alcohol was not used in the extraction procedure by Wilgus. These differences may be due to the possibility that a combined extraction with alcohol and ether is more complete than when ether alone is used. Wilgus (1931) reports bone ash determinations of approximately 50 per cent for normal chicks 16 weeks of age; while some of the normal groups in the present study showed values as high as 55 per cent.

Blood Serum Calcium Determinations.—Serum calcium was determined on a composite sample of blood taken from the four birds of each group used for bone ash determinations. By means of heart puncture, four cubic centimeters of blood were taken from each bird. The blood samples from the four birds of each group were pooled for analysis. Since many of the pullets were in egg production at 24 weeks of age, blood samples were taken only from those birds not in production. The Clark-Collip modification of the Kramer-Tisdall method was used for determining the serum calcium of the samples. The blood calcium determinations are given in Table 3.

Considerable variation exists in reported data on the calcium content of the blood serum of rachitic and of normal chicks. The data here presented indicate the degree of rickets present in each of the 10 groups. The phosphorus content of the blood serum was not determined. Ackerson, Blish, and Musschl (1925) and Bethke, Record, and Kennard (1933) have shown that as the amount of calcium in the blood serum decreases with the development of rickets, the amount of phosphorus also decreases.

TABLE 3. BONE ASH AND BLOOD CALCIUM DETERMINATIONS, 0 TO 24 WEEKS

Group number	1	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	0	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Age in weeks										
8 Per cent ash in tibiae*	39.2	44.6	51.1	51.1	51.2	52.1	52.2	51.7	52.5	52.5
Calcium*	1.8	7.5	9.4	11.1	10.3	11.5	10.5	10.3	10.9	10.4
16 Per cent ash in tibiae*	47.1	50.9	51.0	52.9	52.5	55.0	53.6	52.6	54.7	54.9
Calcium*	5.6	8.6	9.5	10.3	10.6	11.5	12.2	11.1	11.1	12.3
24 Per cent ash in tibiae*	+	49.2	50.5	56.1	54.5	57.0	55.9	54.8	56.8	54.7
Calcium*	+	10.4	11.6	11.8	11.9	11.8	12.0	11.9	11.6	11.9

* Milligrams calcium per 100 cubic centimeters of blood serum.
† Group 1 was discontinued at the end of the sixteenth week.

At eight weeks of age, studies were made of the histological structure of the left tibiae of those birds used in the bone ash determinations. The four bones from Group 1 (no cod liver oil) were diagnosed as being definitely rachitic. One bone in Group 2 (one-thirty-second per cent cod liver oil) was rachitic, two showed slight rachitic lesions, and one showed slight deviations from normal. In Group 3 (one-sixteenth per cent cod liver oil) three bones showed slight deviation from normal and one bone was normal. All bones in the remaining groups were normal.

At 16 weeks, histological bone structure studies showed all bones of Group 1 (no cod liver oil) to be definitely rachitic. Two of the bones in Group 2 (one-thirty-second per cent cod liver oil) were diagnosed as rachitic and two indicated a slight deviation from normal. In the remaining groups the bones were normal. At this time (16 weeks) Group 1 was discarded.

When the pullets were 24 weeks of age, all bones which were examined were diagnosed as normal with the exception of Group 2 (one-thirty-second per cent cod liver oil). One bone in this group was definitely rachitic, two showed slight deviations from normal, and the fourth bone was normal according to histological structure studies. Figures 2 and 3 show representative bone photographs from each group at 8 and 16 weeks respectively. It was not possible to obtain satisfactory photographs of bone structure from birds 24 weeks of age.

Mortality.—For the first 24 weeks of this experiment the mortality was not excessive in any of the groups except Group 1 (no cod liver oil). A large part of the mortality in this group was caused by rickets.

Summary of Results with Growing Chicks

It is evident from the manner in which Group 1 (no cod liver oil) reacted that the basal rations used during the period of growth were definitely rachitogenic. The first external symptoms of rickets in this group were observed when the chicks were three and one-half weeks old.



FIG. 2. HISTOLOGICAL STRUCTURE OF THE LIVER THREE AT EIGHT WEEKS



FIG. 3. HISTOLOGICAL STRUCTURE OF THE LIVER AT 16 WEEKS

By the time they were 10 weeks of age all chicks in this group showed marked external symptoms of rickets. The chicks of Group 2 (one thirty-second per cent cod liver oil) developed noticeable external symptoms of rickets at about seven weeks of age. When 14 weeks old, all showed marked indications of a rachitic condition. Crooked breast bones were quite common. At 24 weeks, the breast bones of all were very crooked. Data on growth, bone ash, and blood calcium indicate that the one thirty-second per cent of the fortified cod liver oil used in this experiment is not sufficient for the development of a normal pullet.

It is doubtful if Group 3 (one-sixteenth per cent cod liver oil) received sufficient oil to protect the pullets against rickets. The histological structure of the bones at 8 and 16 weeks showed a slight deviation from normal, and the low blood calcium values at these same periods indicated a slight rachitic condition (Group 3). Only slight differences were observed in the birds of the five remaining confined groups fed higher levels of cod liver oil as evidenced by growth, bone ash, blood calcium, and general appearance.

At no time during the growing period were external symptoms of rickets apparent in any of the groups except Groups 1 and 2. From hatching time to 24 weeks of age the results indicate that one-eighth per cent of the fortified cod liver oil used, when added to an all mash ration, would be the effective level under the most adverse conditions. When equal parts of grain and mash are fed, the latter should contain one-fourth per cent of an oil equivalent in potency to the one used in this experiment. In the first 24 weeks, each pullet when fed one-eighth per cent of fortified cod liver oil, consumed approximately 3000 International units of vitamin D.

Bethke and associates (1933) found that under the conditions of their experiments the chick required a minimum of approximately seven rat units (18.9 International units) of vitamin D from cod liver oil per 100 grams of ration for normal calcification. In the work here reported, no external symptoms of rickets appeared in Group 3 (one-sixteenth per cent fortified cod liver oil). The ration fed to this group contained 17 International units of vitamin D per 100 grams of ration. These results seem to be in close agreement with the findings of Bethke and coworkers (1933).

Results in Laying Period—24 to 76 Weeks

When the pullets were 24 weeks of age, the number in each group was reduced to 50 representative individuals. At this time, only 49 pullets remained in Group 2 (one thirty-second per cent cod liver oil). The mash used during the period (12 to 24 weeks) also served as the laying mash. No calcium supplements were fed to any of the groups but an insoluble grit was constantly available in hoppers.

Mash consumption was recorded in 4-week intervals (Table 4). The number of pounds of mash consumed per pullet was calculated on a hen-day basis and recorded as the average feed consumption per 100 pullets. Three cockerels were added to each group during the breeding season.

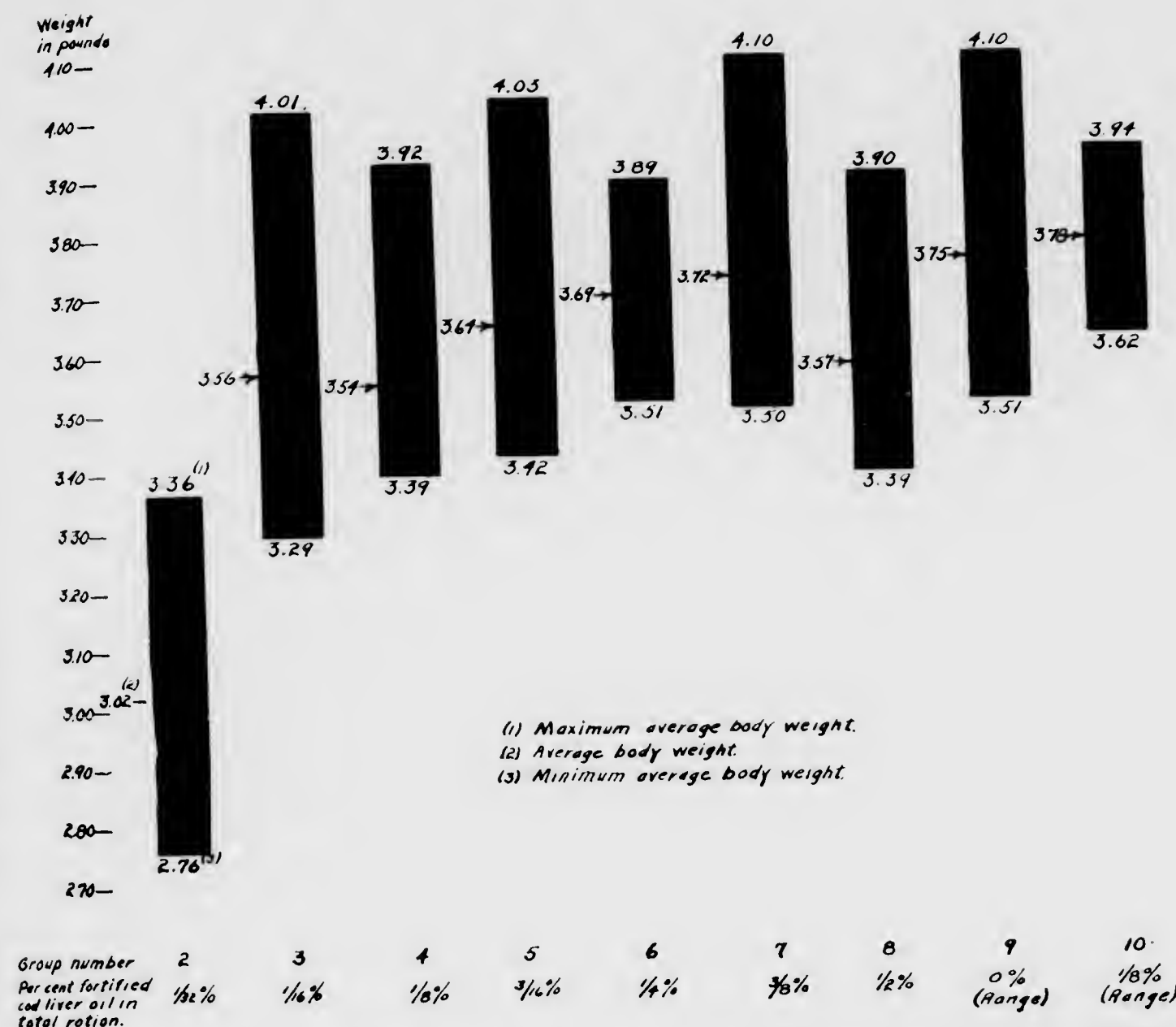


FIG. 4. DEGREE OF VARIATION IN AVERAGE BODY WEIGHT OF PULLETS DURING THE LAYING YEAR

which extended from the 40th through the 60th week. The average number of International units of vitamin D consumed per pullet for each of the four 13-week periods of the laying year is given in Table 5.

TABLE 4. FEED CONSUMPTION

(Average number of pounds of mash consumed per 100 pullets from 24 to 76 weeks)									
Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Weeks									
24-28	422.1	460.7	549.1	591.7	587.2	572.5	566.6	559.5	585.4
28-32	461.8	511.2	569.7	596.3	612.2	607.5	579.1	581.0	593.5
32-36	438.5	458.8	526.6	522.5	597.8	591.5	568.9	601.2	564.8
36-40	419.8	481.6	565.0	659.2	664.6	678.0	694.3	682.2	654.6
40-44	516.8	592.7	672.6	726.2	767.9	698.7	757.3	685.6	711.2
44-48	491.5	580.1	642.4	679.1	628.2	638.1	678.7	677.7	621.4
48-52	571.9	610.7	666.2	683.8	667.0	692.8	679.5	693.1	683.1
52-56	533.1	578.9	679.7	685.4	693.3	703.9	718.9	677.2	654.3
56-60	462.8	551.1	643.3	726.8	670.3	683.3	657.6	600.0	602.0
60-64	482.5	461.3	509.2	587.1	580.4	537.4	555.9	514.4	545.1
64-68	422.7	455.6	556.1	570.5	581.7	542.0	568.9	596.3	590.9
68-72	446.5	459.6	529.0	480.4	551.3	525.0	527.9	525.1	580.3
72-76	437.8	445.6	511.2	533.8	581.1	539.6	503.7	534.2	542.2
Total pounds of mash consumed, 24 to 76 weeks	6013.9	6647.8	7620.2	8042.7	8129.2	8010.1	8057.4	7897.5	7928.5

TABLE 5. VITAMIN D INTAKE

(Average number of International Units from cod liver oil consumed per pullet from 24 to 76 weeks)									
Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Weeks									
24-37	548	1,187	2,735	4,306	6,012	8,915	11,563	0	2,920
37-50	616	1,408	3,172	5,148	6,638	10,065	11,963	0	3,314
50-63	601	1,390	3,120	5,039	6,542	9,813	12,063	0	3,671
63-76	564	1,130	2,639	3,976	5,716	7,995	10,652	0	2,881
Total number of units, 24 to 76 weeks	2,302	5,085	11,666	18,469	24,907	36,788	49,341	0	12,136

Beginning October 1, 1932, artificial morning lights were used in the laying pens. The birds were provided a 12-hour day. Because Pens 2 to 8 inclusive were so constructed as to exclude direct sunlight, it was necessary to illuminate artificially these pens throughout each day. To secure uniformity, similar lighting practices were followed in Pens 9 and 10.

Body Weight of Pullets.—The average body weight of the pullets is given in Table 6. The pullets of each group were individually weighed every four weeks. From the 28th to the end of the 36th week, no group showed an increase in body weight. A rather marked decrease in egg production occurred during the period from 32 to 36 weeks. At 36 weeks, artificial illumination was increased to provide the pullets a 13-hour day. In addition, a portion of the dry mash that the birds ordinarily received was moistened with water to a crumbly consistency and fed daily at noon. It is obvious from the weight of the birds at 40 weeks that these two changes in management had the desired effect. The failure of the pullets to increase in body weight followed by a drop in egg production (Table 7) was due, in part at least, to the fact that a 12-hour day was of insufficient length for proper food intake under the conditions of this experiment. Maximum body weight of the pullets was reached in all groups at 40 weeks of age.

Figure 4 shows the amount of variation that existed in the body weights of the pullets in the various groups during the laying year. The average of the body weights for 28 to 76 weeks is indicated on the graph by an arrow. Above the arrow is plotted the maximum and below the minimum average body weight attained by the pullets of each group during the year. The average body weight increased as the per cent of cod liver oil increased. A small degree of variation existed in the average body weights of the pullets in Groups 6 and 10. Although there was more variation in Groups 7 and 9, the minimum average body weight was similar to that of Group 6.

Egg Production.—All pullets were trapnested throughout the laying year. Calculations determining the per cent egg production and num-

TABLE 6. AVERAGE BODY WEIGHT OF PULLETS IN POUNDS, 28 TO 76 WEEKS

Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Age in Weeks									
28	2.88	3.61	3.61	3.77	3.75	3.80	3.61	3.80	3.78
32	3.04	3.61	3.52	3.72	3.64	3.78	3.55	3.80	3.82
36	3.05	3.53	3.47	3.64	3.66	3.71	3.45	3.82	3.68
40	3.36	4.01	3.92	4.03	3.88	4.10	3.90	4.10	3.94
44	3.12	3.82	3.73	3.81	3.89	3.90	3.81	3.88	3.88
48	3.08	3.80	3.49	3.63	3.73	3.74	3.62	3.80	3.82
52	3.45	3.70	3.50	3.69	3.71	3.67	3.64	3.88	3.87
56	2.97	3.47	3.55	3.61	3.81	3.73	3.60	3.78	3.83
60	2.87	3.36	3.39	3.55	3.67	3.68	3.49	3.61	3.62
64	2.76	3.37	3.46	3.49	3.55	3.62	3.45	3.58	3.64
68	2.84	3.29	3.41	3.42	3.51	3.52	3.39	3.51	3.70
72	3.08	3.31	2.49	3.41	3.59	3.58	3.41	3.55	3.75
76	3.09	3.43	3.56	3.51	3.55	3.50	3.39	3.60	3.77
Average, 28 to 76 weeks	3.02	3.56	3.54	3.64	3.69	3.72	3.57	3.75	3.78

ber of eggs laid per pullet were made on a hen-day basis. A rather marked decrease in egg production occurred between the 32nd and 40th weeks (Table 7). After the length of day had been increased and moist mash fed, egg production increased rapidly. The average number of

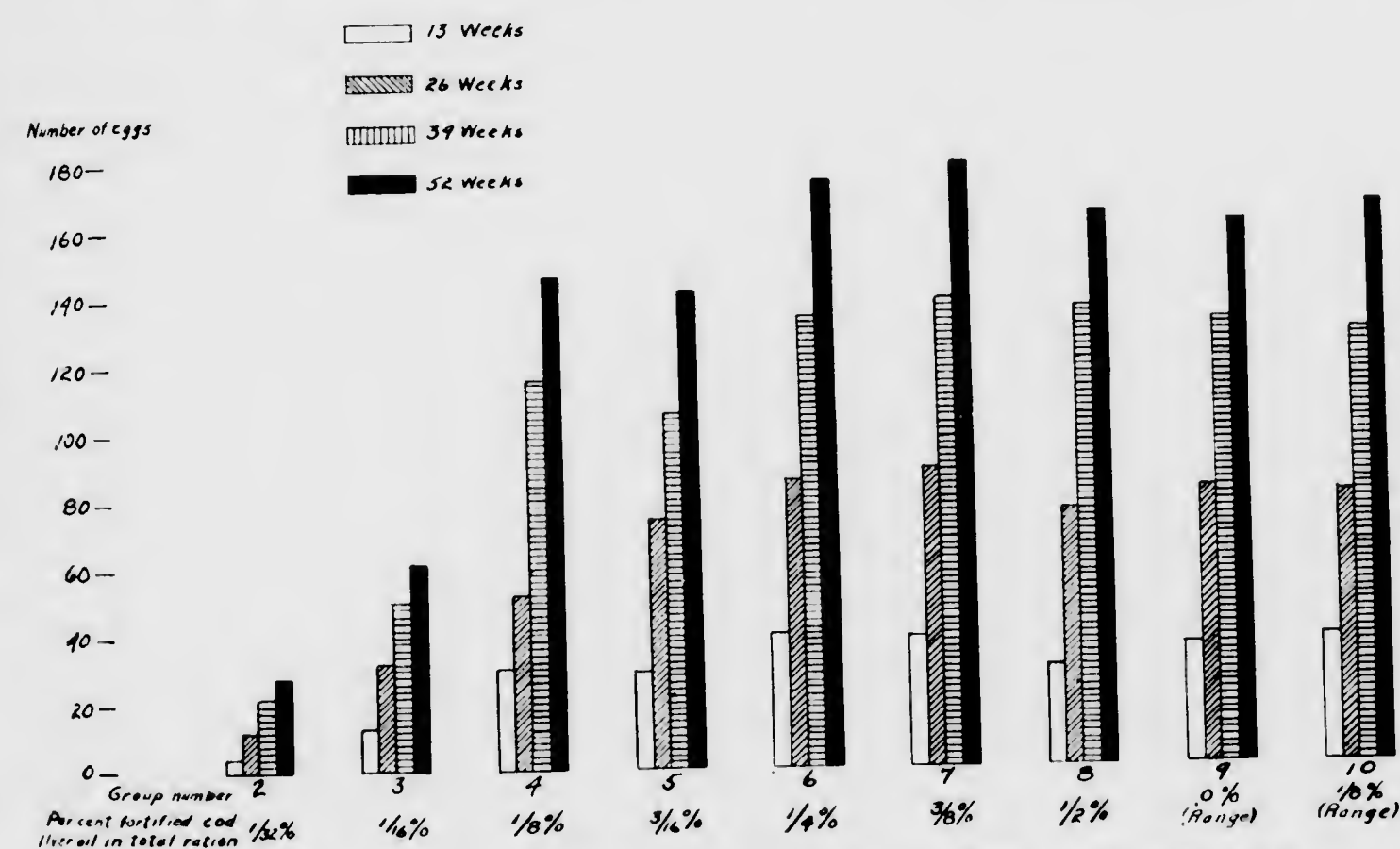


FIG. 5. AVERAGE NUMBER OF EGGS LAID PER PULLET FOR 13, 26, 39, AND 52-WEEK PERIODS

eggs laid per pullet during the four quarterly periods of the laying year is shown graphically in Figure 5. The first 13 weeks of production extended from September 27 to December 26, 1932; 26 weeks were completed on March 27, 1933; 39 weeks on June 26, 1933; and the laying year was completed on September 25, 1933.

TABLE 7. AVERAGE PER CENT EGG PRODUCTION

Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Weeks									
24-28	5.3	14.1	40.4	36.9	34.4	40.9	31.2	35.9	49.5
28-32	5.1	23.8	43.6	44.6	57.3	53.2	47.1	47.6	51.5
32-36	1.5	6.8	19.4	19.2	43.0	37.8	25.8	38.1	30.2
36-40	3.2	8.9	30.0	24.4	37.8	35.4	26.6	36.1	24.1
40-44	6.6	23.0	59.3	62.2	59.1	65.0	67.3	57.1	55.7
44-48	10.4	23.4	42.7	55.7	55.2	58.6	54.0	54.8	48.5
48-52	24.0	25.1	47.6	41.7	40.9	51.8	46.0	54.8	54.7
52-56	21.7	27.2	53.9	47.4	58.2	59.2	63.5	61.8	61.2
56-60	8.3	16.7	54.1	51.8	60.3	64.9	62.6	56.0	58.4
60-64	7.6	12.7	35.9	38.3	47.5	43.7	47.0	39.2	38.2
64-68	3.1	11.4	33.9	34.6	45.2	45.4	51.1	33.3	49.9
68-72	0.8	10.3	28.6	16.1	40.2	41.3	40.7	29.7	43.0
72-76	8.0	5.3	23.0	14.4	33.5	39.5	24.5	13.5	32.1
Average, 24 to 76 weeks	7.6	16.6	39.9	38.6	47.4	48.8	44.7	44.0	45.4

Table 7 and Figure 5, indicate that Groups 6 and 7, fed one-fourth and three-eighths of one per cent of the fortified cod liver oil respectively, were superior in egg production. Other investigators, as Parkhurst (1930), Graham and coworkers (1931), Hendricks and associates (1931), and Edson (1932), have shown that the addition of cod liver oil to otherwise well-balanced laying rations increases egg production. The present study shows that a close correlation exists between the vitamin D factor in the cod liver oil fed and egg production.

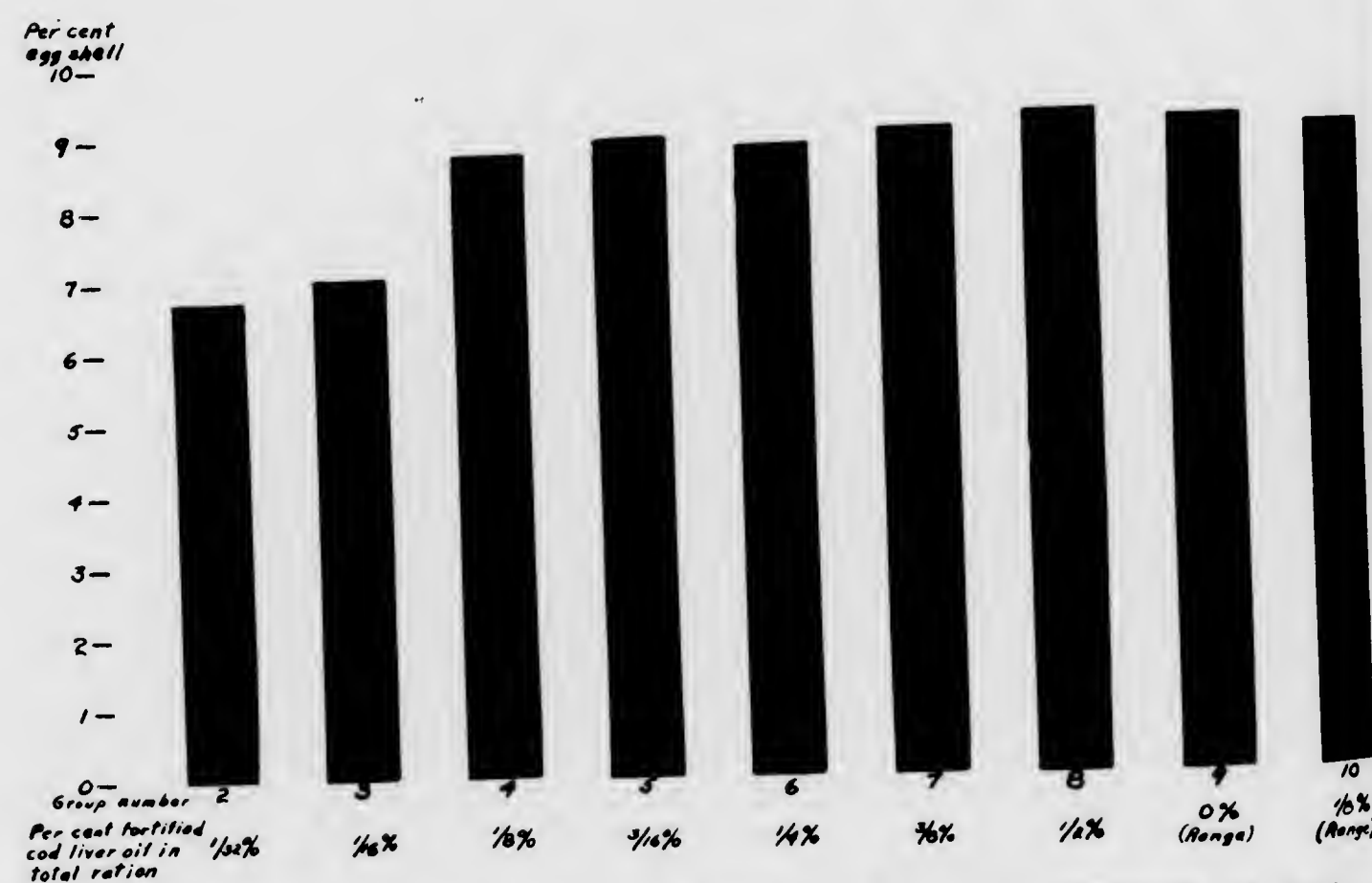


FIG. 6. AVERAGE PER CENT EGG SHELL OF TOTAL WEIGHT OF EGG

Weight of Eggs.—The total daily egg production, by groups, was weighed. The average weight is given in Table 8. It appears that adequate amounts of vitamin D tend to increase egg size. Our studies indicate that when sufficient amounts of vitamin D are supplied, a heavier egg shell is produced (Fig. 6). Hendricks and associates (1931) conclude, "Feeding cod liver oil or administering ultra-violet irradiation to laying hens confined without access to direct sunlight or green feed increased egg production and the thickness of egg shell and improved the general condition of the birds. Both of these vitamin supplements also show indications of a tendency to increase egg weight and improve the hatchability of the eggs." Parkhurst (1933) states that pullets housed behind ordinary glass and given inadequate amounts of vitamin D gave significantly lower average egg weights than comparable pens given adequate quantities of the antirachitic vitamin.

TABLE 8. AVERAGE WEIGHT OF EGGS IN GRAMS *

Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Weeks									
24-28	43.8	43.5	44.8	45.0	45.9	45.5	45.6	45.8	45.7
28-32	46.2	46.1	47.3	47.6	48.1	48.2	47.5	48.8	48.8
32-36	40.5	48.0	49.1	48.9	49.9	50.6	50.0	50.6	50.2
36-40	46.9	49.5	52.2	52.3	52.9	53.6	51.5	53.0	53.3
40-44	51.1	52.1	54.6	54.4	54.9	55.6	54.5	54.1	54.2
44-48	50.8	50.9	52.9	54.5	54.1	53.8	53.9	55.0	54.1
48-52	54.9	53.5	53.8	51.8	55.6	54.5	54.2	55.0	55.4
52-56	54.6	53.8	54.1	55.4	55.3	54.5	54.3	54.9	54.5
56-60	43.6	53.1	53.8	54.7	54.8	54.3	53.5	54.0	53.7
60-64	47.6	52.6	51.8	53.6	53.2	51.9	52.4	54.9	54.9
64-68	50.0	51.8	52.6	54.4	53.8	53.6	53.5	54.3	54.9
68-72	56.7	54.8	53.6	55.6	54.9	55.2	54.5	56.0	55.3
72-76	53.3	58.6	55.2	57.5	56.2	56.8	55.2	56.3	56.6
Average, 24 to 76 weeks	51.0	50.9	51.7	52.7	52.3	52.7	52.3	53.1	52.9

* A 2-ounce egg weighs 56.7 grams.

The averages shown in Fig. 6 were obtained from 10 eggs of each group at 12 times during the laying year. The eggs were weighed individually on analytical balances the day they were laid. After weighing, the eggs were broken and the albumin washed out with distilled water. The shells from the 10 eggs of each group were pooled, pulverized in a mortar, dried to a constant weight, and weighed. A 2-gram sample from each composite sample was ashed. From this ash determination, the per cent shell ash of the total weight of the 10 eggs was calculated (Table 9). These results further substantiate the degree of correlation that exists between the vitamin D intake of laying pullets and the quantity and quality of egg shell produced.

Blood Serum.—The results of 12 calcium determinations of the blood serum made during the year in each of the groups are given in Table 10. These determinations were made on a composite sample of blood from

TABLE 9. PER CENT SHELL ASH OF TOTAL WEIGHT OF EGG

Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Age in weeks									
28	3.3	4.1	5.1	5.2	5.3	5.2	5.2	5.4	5.3
32	3.5	4.0	5.0	5.1	5.0	5.3	5.5	5.2	5.3
36	3.7	3.9	5.3	4.9	5.0	5.2	4.7	5.0	4.7
40	3.2	4.1	4.9	4.9	5.1	5.3	5.2	4.9	5.1
44	3.3	3.5	4.3	4.7	4.7	4.7	5.1	4.7	4.7
48	4.3	3.7	4.8	4.9	4.7	4.8	4.9	4.7	4.6
52	3.7	3.8	4.8	4.8	4.7	4.8	4.7	4.8	4.8
56	3.2	3.6	4.5	4.6	4.4	4.1	4.6	4.6	4.5
60	*	3.5	4.2	4.6	4.4	4.6	4.7	4.4	4.5
64	*	3.5	4.0	4.2	4.5	4.2	4.7	4.7	4.6
68	*	3.2	3.8	4.4	4.4	4.5	4.8	4.6	4.6
72	3.5	3.6	4.7	4.8	4.4	4.6	4.9	4.8	4.6
Average, 28 to 72 weeks	3.5	3.7	4.6	4.8	4.7	4.8	4.9	4.8	4.8

*At the time this determination was made a sufficient number of eggs was not obtainable from Group 2 for this purpose.

five birds of each group. All birds from which samples of blood were taken were in production. Four cubic centimeters of blood were removed from pullets in egg production. This was done with a hypodermic syringe inserted into the brachial vein of the left wing. The serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method. The serum calcium content of the blood varied considerably throughout the laying period. The average of the 12 serum calcium determinations showed that only slight differences existed among the various groups, except for Groups 2 and 3 which were fed low levels of cod liver oil. The serum calcium content of the blood from the pullets in these two groups was low.

TABLE 10. BLOOD SERUM CALCIUM DETERMINATIONS, 28 TO 72 WEEKS (Milligrams calcium per 100 cubic centimeters of blood serum)

Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Age in weeks									
28	19.0	18.1	20.4	20.4	22.5	24.4	25.1	27.3	22.6
32	17.4	21.4	25.2	26.2	21.9	21.6	23.5	22.6	22.6
36	17.1	21.3	25.8	27.0	25.1	27.4	30.3	25.5	25.3
40	22.3	22.8	22.6	23.3	23.4	25.4	23.2	27.7	27.6
44	20.3	22.3	26.6	28.5	27.6	29.8	30.0	28.0	27.4
48	21.0	20.6	27.6	26.5	27.2	28.0	27.4	30.3	28.8
52	15.2	21.8	27.4	28.6	29.0	29.6	28.7	26.1	28.9
56	21.1	19.9	26.5	24.0	24.6	25.0	20.2	27.4	25.8
60	14.6	27.6	21.9	23.9	26.6	25.0	22.7	28.3	22.4
64	*	18.8	21.0	20.8	25.2	25.7	27.3	28.0	27.0
68	*	17.0	19.8	22.6	20.1	23.4	19.0	20.9	21.8
72	18.6	25.1	26.6	24.8	27.0	23.8	25.4	27.5	25.6
Average, 28 to 72 weeks	18.7	21.4	24.3	24.7	25.5	26.0	25.2	26.6	25.5

*At the time this determination was made sufficient birds were not in production to obtain a sample of blood.

Mortality.—During the latter part of this experiment, the mortality was rather excessive particularly in Group 2 (one thirty-second per cent cod liver oil). There was no significant difference in mortality among the other groups.

Hatchability.—Hatchability data were secured from the pens fed various levels of cod liver oil. Five settings of eggs from each of the nine groups of pullets were incubated. The hatching period extended from March 10, 1933 to June 16, 1933. Three cockerels were placed in each group of pullets three weeks prior to the time hatching eggs were saved. The cockerels were rotated from pen to pen twice weekly.

TABLE 11. HATCHING RESULTS

Group number	2	3	4	5	6	7	8	9	10
Per cent fortified cod liver oil in total ration	1/32	1/16	1/8	3/16	1/4	3/8	1/2	0 (Range)	1/8 (Range)
Hatch Date	Number of eggs set								
hatched No.									
1 March 10, 1933	5	46	100	100	100	100	100	100	100
2 April 7, 1933	41	86	100	100	100	100	100	100	100
3 May 12, 1933	61	80	100	100	100	100	100	100	100
4 June 1, 1933	18	34	100	100	100	100	100	100	100
5 June 16, 1933	6	38	100	100	100	100	100	100	100
Total, 5 hatches	131	284	500	500	500	500	500	500	500
	Per cent fertility								
1 March 10, 1933	80.0	91.3	90.0	88.0	85.0	81.0	61.0	79.0	87.0
2 April 7, 1933	95.1	82.6	82.0	85.0	82.0	76.0	75.0	81.0	89.0
3 May 12, 1933	91.8	81.3	72.0	81.0	86.0	93.0	75.0	79.0	92.0
4 June 1, 1933	77.8	91.2	74.0	77.0	88.0	77.0	62.0	86.0	87.0
5 June 16, 1933	66.7	76.3	84.0	75.0	81.0	81.0	62.0	77.0	89.0
Average, 5 hatches	89.3	83.8	80.4	81.2	85.0	82.2	67.0	80.4	88.8
	Number of chicks hatched								
1 March 10, 1933	1	17	79	71	76	73	59	69	80
2 April 7, 1933	27	29	50	69	65	70	60	76	76
3 May 12, 1933	37	25	51	73	69	86	64	68	70
4 June 1, 1933	1	13	56	67	80	67	55	78	75
5 June 16, 1933	0	12	61	63	71	66	56	75	69
Total, 5 hatches	66	106	300	343	361	362	294	366	370
	Per cent hatch of fertile eggs								
1 March 10, 1933	25.0	40.5	87.8	80.7	89.4	90.1	96.7	87.3	92.0
2 April 7, 1933	24.2	74.9	61.0	81.2	79.3	92.1	80.6	93.8	85.4
3 May 12, 1933	66.1	38.5	70.8	90.1	80.2	92.5	85.3	86.1	76.1
4 June 1, 1933	7.1	41.9	75.7	87.0	90.9	87.0	88.7	90.7	86.2
5 June 16, 1933	0.0	41.4	76.2	84.0	81.5	78.6	90.3	97.4	77.5
Average, 5 hatches	56.4	44.5	74.6	84.5	84.9	88.1	87.8	91.0	83.3

during the breeding season. The eggs for incubation purposes were individually pedigreed. Hatching eggs were saved for one week previous to setting and, so far as possible, three eggs and never more than five were set from each pullet. For each hatch, 100 eggs from each group of pullets were incubated, with the exception of Groups 2 and 3, each of which produced less than this number of eggs during the period. Eggs laid nearest to the time of setting were selected for incubation purposes. The results of the five hatches are given in Table 11. Fertility was high in all groups except Group 8 (one-half per cent cod liver oil). For some unknown reason, fertility in this group was consistently low in each of the five hatches. There was a degree of correlation between the amount of vitamin D supplied to breeders in the form of fortified cod liver oil and the per cent hatch of fertile eggs.

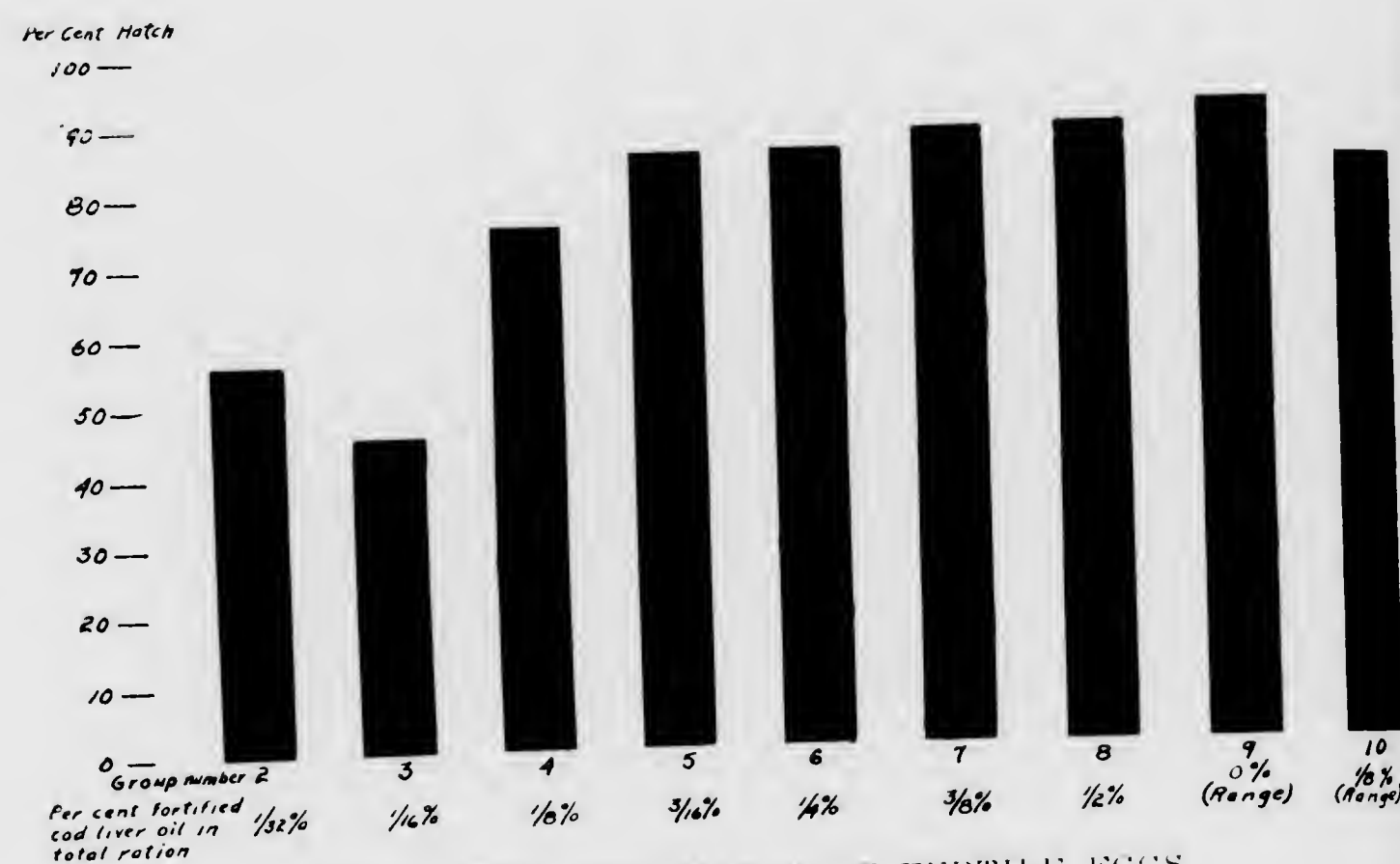


FIG. 7. PER CENT HATCH OF FERTILE EGGS
(Summary of five hatches)

In a 3-year study of the effect of various poultry rations on the hatching power of hen eggs, Graham and associates (1931) found that cod liver oil added to various combinations of animal protein feeds increased the hatchability of the eggs produced. Edson (1932) after three years of work, concluded that hatchability was improved considerably when cod liver oil was fed to confined pullets. The per cent hatch of fertile eggs from five hatches, involving 500 eggs from each group except Groups 2 and 3, is shown in Figure 7.

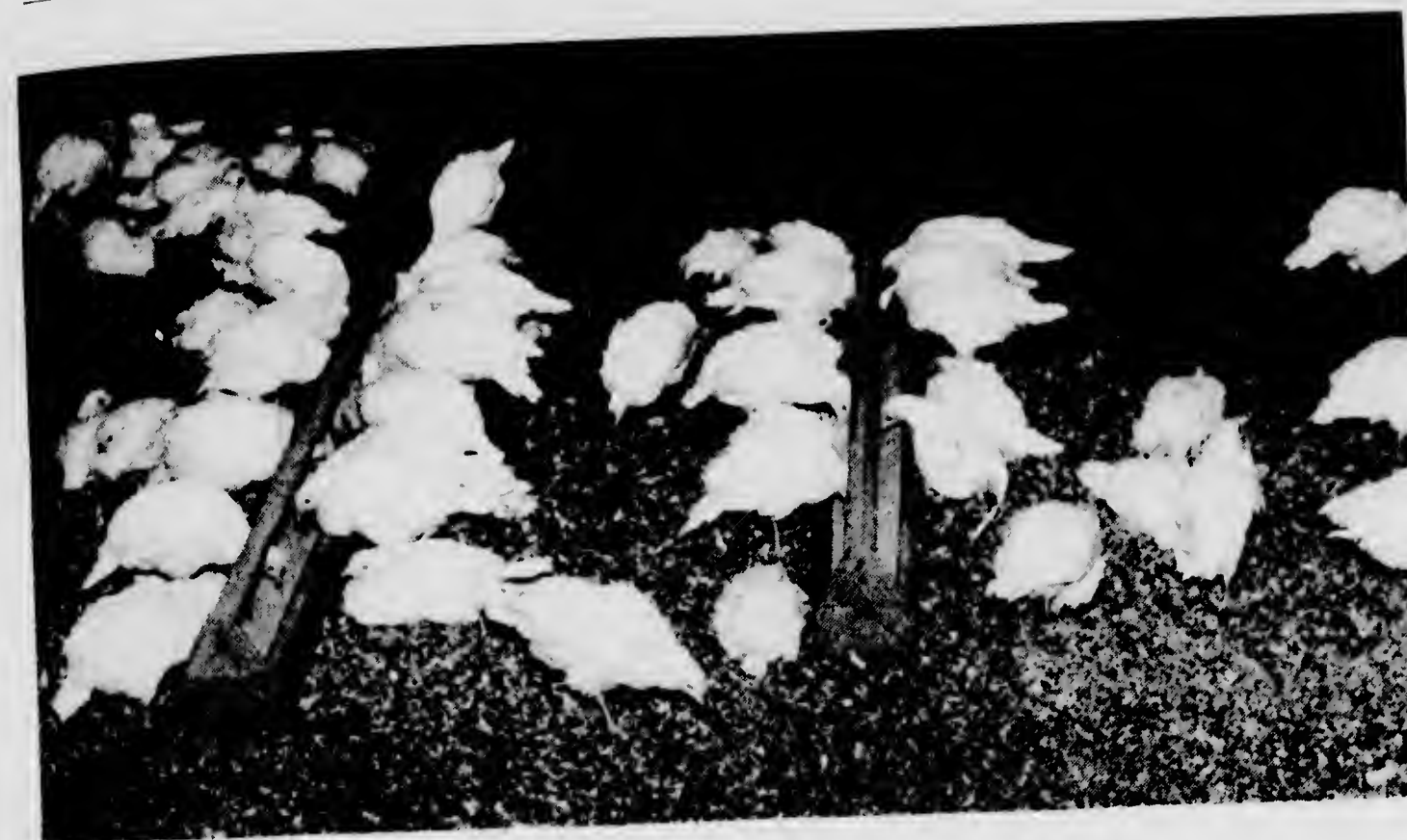


FIG. 8. GROUP 1, WHEN EIGHT WEEKS OLD
These chicks received no oil

Summary of Results With Laying Pullets

Egg production was not satisfactory in Groups 2, 3, 4, and 5 which were fed one thirty-second, one-sixteenth, one-eighth, and three-sixteenths of one per cent of a fortified cod liver oil respectively. When all factors of measurement were considered, Groups 2 and 3 were far inferior to

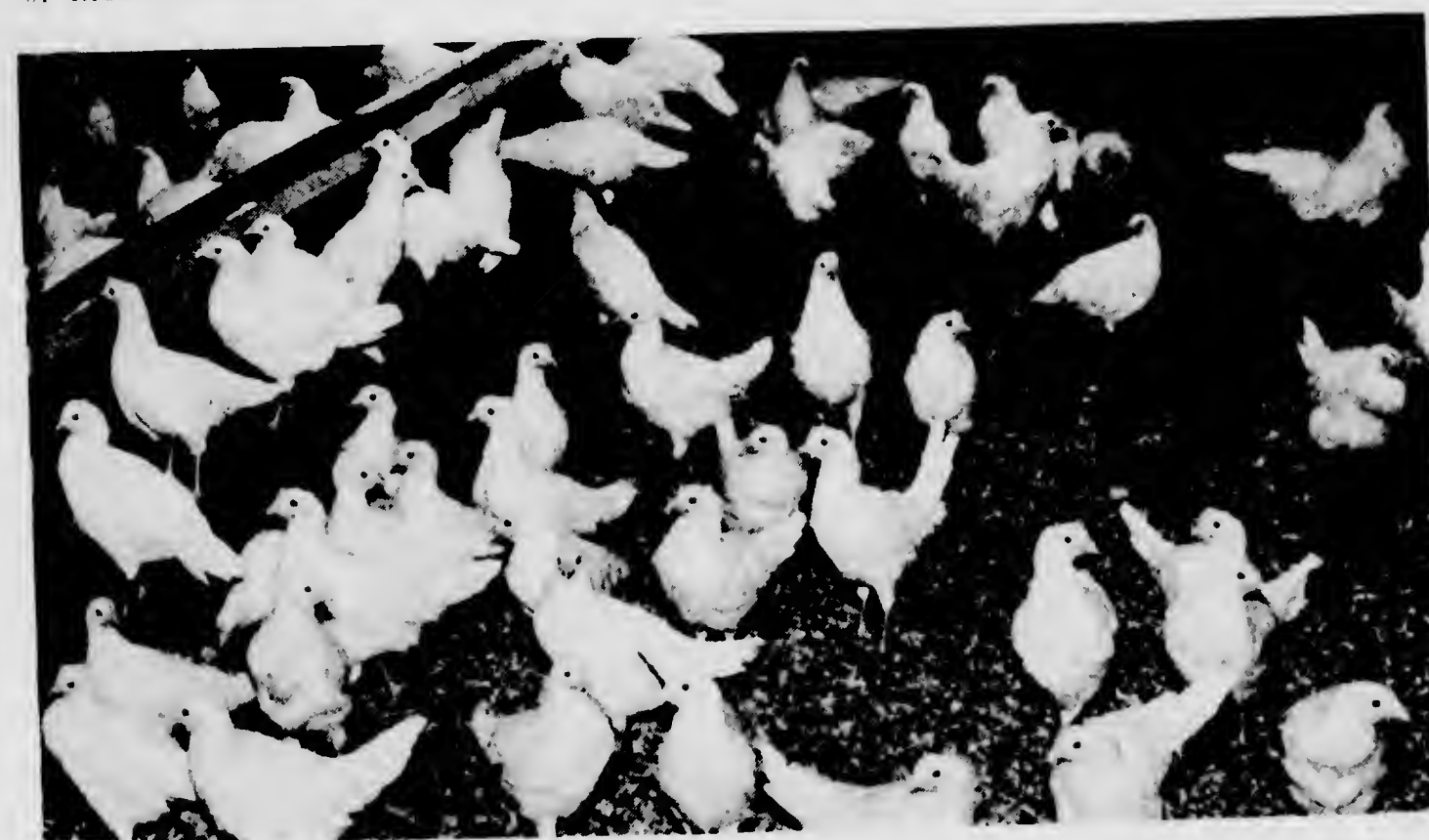


FIG. 9. GROUP 4 AT EIGHT WEEKS OF AGE
These chicks received one-eighth of one per cent fortified cod liver oil

any of the other groups. In maintenance of body weight, egg production, egg weight, per cent egg shell, and calcium content of blood, no apparent significant differences existed between Groups 6 and 7 which were fed one-fourth and three-eighths of one per cent of cod liver oil respectively. Satisfactory results were obtained from both of these groups whereas, in the case of Group 8 (one-half per cent of cod liver oil), the results were not so favorable. Groups 9 and 10, in which the birds had access to range at all times, exhibited only small differences. The evidence indicates that when laying pullets are denied access to direct sunlight, one-fourth per cent of a fortified cod liver oil or its vitamin D equivalent (270 International units of vitamin D per gram) in the total ration is the most effective level. This amount will supply sufficient vitamin D to fulfill adequately the requirements of laying pullets maintained under the most adverse conditions. When laying pullets subjected to environmental conditions similar to those employed in this study are fed equal parts of mash and grain, the mash should contain one-half of one per cent of a fortified cod liver oil of equal potency.

Effect on the Offspring

To determine the effect on the offspring when varying levels of cod liver oil were fed to the mother hen, all normal chicks from hatches 2, 3, and 5 (Table 11) were brooded for a period of eight weeks. Hatches 1 and 4 were not brooded but were used only for hatchability studies. The chicks from hatches 2, 3, and 5 were weighed by groups at hatching time and bi-weekly thereafter for a period of eight weeks.

The same basal ration that was fed to the parent stock during their brooding period was fed to the progeny. All groups of hatch 2 received,



FIG. 10. GROUP 1, 16 WEEKS OLD, RECEIVED NO OIL AND HAD NO RANGE
Compare with figure on front cover

in addition to the basal ration, one-sixteenth of one per cent of the cod liver oil fed to the parent stock which had been found in the work of the previous year to be the minimum protective level. No cod liver oil supplement was fed to any of the lots of hatch 3. The level of cod liver oil fed the parent stock was also supplied to all groups of hatch 5. All groups of hatches 2 and 3 and Groups 3 to 8 inclusive of hatch 5 were placed in battery brooders and deprived of direct sunshine. In hatch 5, Groups 9 and 10 were brooded in colony houses with access to range.

Data were obtained on growth, feed consumption, mortality, blood calcium, and bone ash of 1551 progeny. There was no evidence that the vigor of the chicks was influenced by the vitamin D intake of the parent stock. The results indicate that in order to determine the degree to which vitamin D is transmitted from the parent to the offspring more sensitive methods of measurement are needed.

General Summary

1. Single Comb White Leghorn chicks, fed an all-mash ration deficient in vitamin D but adequate in all other known food essentials, and reared in battery brooders and deprived of sunshine, developed rickets at three and one-half weeks of age.
2. For Single Comb White Leghorn chicks deprived of sunshine from hatching time to 24 weeks of age, the minimum protective level of a fortified cod liver oil (270 International units of vitamin D per gram) was one-sixteenth of one per cent of the total ration.
3. Very satisfactory results in growth and bone development were obtained when one-eighth of one per cent of this oil was used.
4. Of the two groups given access to range during the normal spring brooding period, no measurable differences were noted between the group receiving no vitamin D supplement and the one supplied with one-eighth of one per cent of a fortified cod liver oil (270 International units of vitamin D per gram).
5. Single Comb White Leghorn pullets confined without access to sunshine gave unsatisfactory egg production when fed an all-mash ration which contained three-sixteenths of one per cent or less of the fortified cod liver oil used in this study.
6. Single Comb White Leghorn laying pullets confined without access to sunshine and fed one-fourth of one per cent of a fortified cod liver oil (270 International units of vitamin D per gram) from time of hatch gave satisfactory results in the maintenance of body weight, egg production, size of egg, quality of egg shell, and hatchability.
7. No measurable differences were observed between two groups of fowls, both of which had access to free range, the one fed from hatching

time through the first year of egg production one-eighth of one per cent of a fortified cod liver oil (270 International units of vitamin D per gram) and the other fed no cod liver oil.

8. For satisfactory performance of Single Comb White Leghorn pullets, as measured by maintenance of body weight, egg production, size of egg, quality of egg shell, blood calcium, and hatchability, a yearly intake of approximately 25,000 International units of vitamin D per pullet was found to be necessary.

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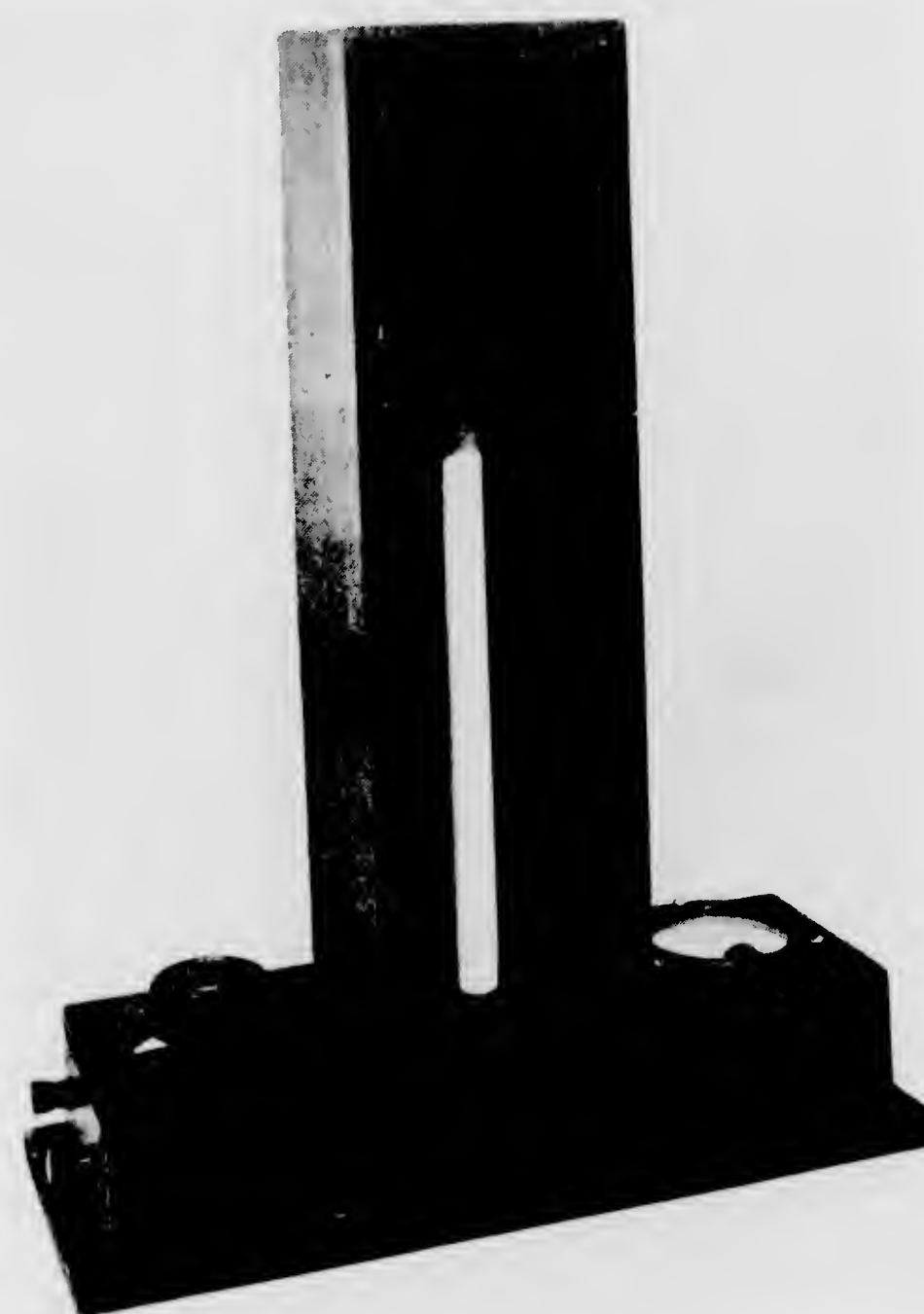
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A Simplified Method for the Rapid Determination of Lead Residues on Apples

TECHNICAL BULLETIN



PHOTOELECTRIC COLORIMETER

THE PENNSYLVANIA STATE COLLEGE
SCHOOL OF AGRICULTURE AND EXPERIMENT STATION
STATE COLLEGE, PENNSYLVANIA

A Simplified Method for the Rapid Determination of Lead Residues on Apples*

DONALD E. H. FREAR AND D. E. HALEY

ACCURATE determination of small amounts of lead on fruit as spray residues has become increasingly important in recent years, because of the interest of the consuming public, and the regulations set up by official bodies charged with the control of food products. This problem is of special concern to the fruit grower, since an early estimate of the amount of spray residue on his fruit will determine his course of action. If the quantity of residue exceeds the tolerance allowed by regulatory agencies, either Federal or State, or both, it becomes necessary to reduce the amount before the fruit is marketed. The usual commercial practice is to wash the fruit.

Existing methods for the determination of lead in spray residues are, in general, characterized by a complicated technique. This makes the determination lengthy and laborious, and requires considerable skill on the part of the operator to secure accurate results. The investigations here reported have had two purposes: to confirm the practicability of the use of the Photronic cell for the determination of small amounts of lead in spray residues, as described by Samuel and Shockey (1); and to propose certain modifications of this general method by which it may be possible to determine the amount of lead in the spray residue accurately and rapidly. The following method appears to fulfill these requirements.

The Apparatus and the Method

The instrument used in this study is considerably different from that described by Samuel and Shockey. The essentials of the apparatus are:

A source of light, in this case a 6-volt automobile headlight bulb of 32-32 candlepower, both filaments of which are connected in series to a 12-volt battery, with a suitable rheostat included in the circuit to vary the intensity of the light.

A photo-electric cell, the most convenient type of which has been found to be the Weston Photronic cell 594, connected to a microammeter which may have a total capacity of 100 or 200 microamperes.

A Nessler tube approximately 25 cm. long and 3 cm. in outside diameter. In this tube is placed the solution to be tested, through which all of the light reaching the Photronic cell must pass.

A sketch of the complete apparatus is shown in Fig. 1. The relatively intense source of light may, in some cases, develop too great heat, which may be dissipated by a water jacket placed about the socket. A hole 1 mm. smaller in diameter than the outside diameter of the

Nessler tube is made in the false bottom of the light chamber directly over the center of the Photronic cell. A tight fit at this point excludes all extraneous light from the cell, and no door is necessary at the front of the apparatus.

The instrument is standardized by taking definite amounts of a solution of C. P. lead acetate corresponding to 0.005, 0.010, 0.015 and 0.020 grains of lead per pound of the apples to be analyzed. When using an

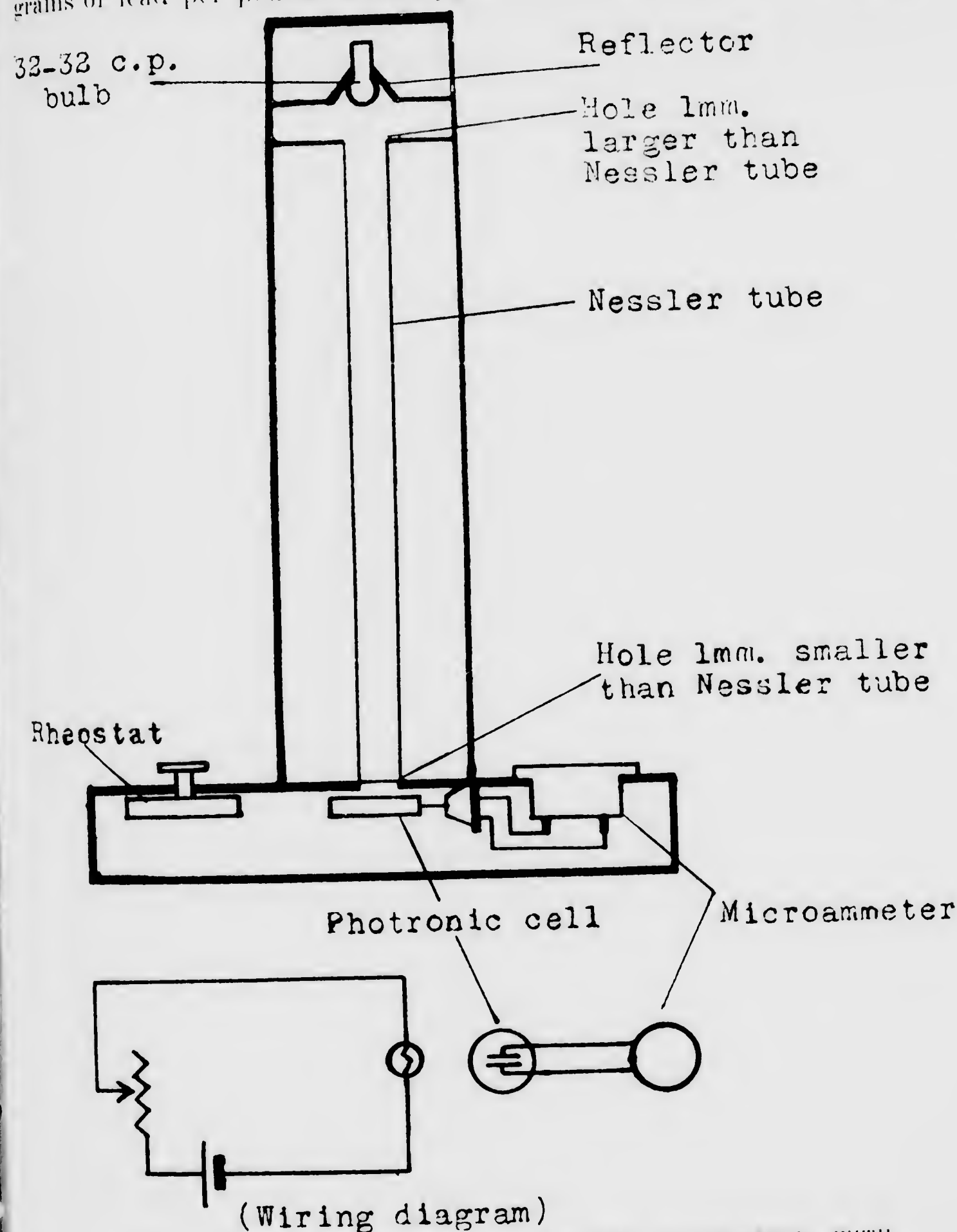


FIG. 1. SECTION THROUGH PHOTOELECTRIC COLORIMETER, WITH WIRING DIAGRAM

* Publication authorized March 2, 1931.

aliquot of the washings representing 140 grams of apples, as will be described later, the corresponding amounts of lead are 0.0001, 0.0002, 0.0003 and 0.0004 grams. To these solutions are added 0.25 gram of sucrose, and 10 ml. of concentrated sulphuric acid. Each of these solutions is boiled until the organic matter chars, and 5 ml. portions of concentrated nitric acid are added until, after boiling, the solution appears clear and colorless, and has a volume of approximately 10 ml.

This solution is diluted when cold with 50 ml. of distilled water and again allowed to cool, and neutralized with 40 ml. of a solution containing 913 ml. of concentrated ammonium hydroxide, 5 grams of potassium cyanide and 31.3 grams of citric acid per liter of solution (2). When cooled to room temperature, this solution is placed in the Nessler tube, made to 100 ml., thoroughly mixed, and the tube is placed in the position in the instrument. The light is adjusted by means of the rheostat so that the microammeter reading is at the maximum of the instrument. Six drops of a 10 per cent solution of sodium sulphide are then added, and the whole mixed and returned to the colorimeter. The reading is taken and a graph prepared, plotting the readings in microamperes against lead concentration in grains per pound. A typical standardization curve is shown in Fig. 2.

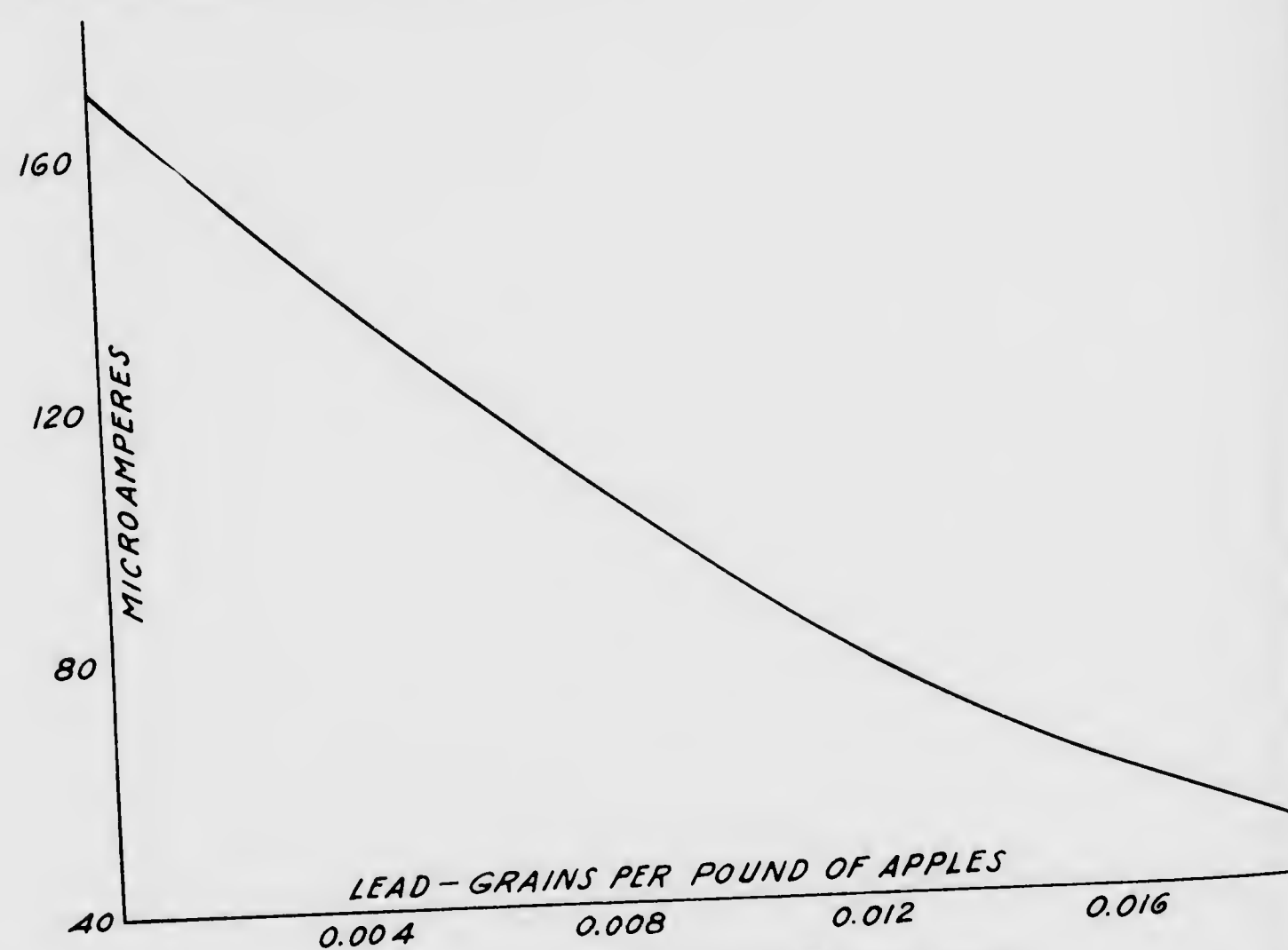


FIG. 2. TYPICAL STANDARDIZATION CURVE

For the determination of lead in spray residues, a 700-gram sample of apples or other fruit to be analyzed is washed with a solution containing 3 per cent by volume of concentrated hydrochloric acid and 1 per cent of sodium chloride. Each fruit is immersed in the solution

for two minutes at a temperature of approximately 95° C. When removed, the calyx and stem ends are thoroughly scrubbed with a rubber policeman and rinsed well with an additional portion of the hot wash solution.

After each fruit in the sample has been washed, the combined washings are transferred to a 500 ml. volumetric flask and made to volume when cool. Aliquot portions of this solution may be used for the determination of arsenic by the modified Gutzeit method. For the determination of lead, an aliquot is taken, and the organic matter is digested with sulphuric acid and nitric acid. The amount of lead present determines the volume of the aliquot taken. For residues containing less than 0.02 grain of lead per pound of apples, an aliquot of 100 ml., representing 140 grams of apples, is satisfactory. The remainder of the procedure is carried out as described in a preceding paragraph. When the final reading is obtained, comparison with the graph (obtained by standardizing the instrument) indicates the amount of lead directly.

Temperature changes affect the output of the cell. This is especially marked when an instrument of low resistance is used in the circuit. When the temperature of the instrument is subject to variation, separate calibration curves may be made at various temperatures.

Several elements may interfere with the determination, thus leading to erroneous results. In the course of ordinary work, tin and mercury, two elements which interfere most seriously, never are encountered. Iron, when present in large amounts, may give readings which are too high. However, using the procedure outlined, analyses for iron indicate that the average total quantity of this element present is negligible. A small amount of sodium metabisulphite may be added as recommended by Scott (2) if iron is present in the reagents.

Tests of the Method

The efficiency of washing in the removal of spray residues has been discussed by several workers (4, 5, 6), but chiefly with the purpose of evolving a method for the removal of residues from marketable fruit, and not from the point of view of quantitative removal on a laboratory scale. Obviously fruit intended for ultimate sale cannot be subjected to extremely rigorous washing procedures, as is possible in the laboratory on a small sample of fruit to be analyzed. Pettey (3) and several other investigators have shown that even at relatively low temperatures a wash solution containing hydrochloric acid is extremely efficient in removing the residue. The addition of an electrolyte, in this case sodium chloride (4, 5), increases the efficiency of the wash.

In order to test the completeness of the removal of the spray residue from the surface of the apple by the washing procedure here applied, several samples of apples were selected to show various amounts of spray residues. Some were taken directly from the trees. In order to secure the higher concentrations it was necessary to spray some apples directly with a suspension of lead arsenate. To this spray was added lime-sulphur mixture, in order that the conditions might be compar-

able with those found in the orchard. The effect of other materials sometimes used in spray mixtures on the efficiency of the washing has not been tested. The apples used in this test were all of the Stayman variety, which has been shown by Hodgkiss (7) to retain spray residues more tenaciously than some other varieties.

The apples were weighed and washed as described above. After washing, the peelings were removed and the stem and calyx ends cut out. The peelings and the cone-shaped end cuts then were digested with nitric acid and sulphuric acid and analyzed for lead. The results are shown in Table 1.

TABLE 1. EFFICIENCY OF THE WASH SOLUTION CONTAINING ONE PER CENT SODIUM CHLORIDE BY WEIGHT AND THREE PER CENT HYDROCHLORIC ACID BY VOLUME

Sample No.	Lead in washings grains per pound	Lead remaining on apple surface grains per pound
1	0.010	0.001
2	0.010	0.001
3	0.013	0.001
4	0.042	none
5	0.071	none
6	0.105	0.001

The accuracy of the determination of lead was ascertained in two ways: first, by a series of recovery tests on solutions of apple washings, using known amounts of lead; and second, by a direct comparison of the results obtained by the proposed method with results secured by the method proposed by the Food and Drug Administration (8), dated February 15, 1933.

In conducting the recovery tests, a large volume of solution obtained by washing apples by the method here described was thoroughly mixed, and the lead determined by analysis, using the method here proposed. Definite amounts of lead as lead acetate were then added to aliquot portions of the original wash solution, the solution again analyzed, and the amounts of lead recovered calculated. The results are given in Table 2.

A comparison between the results obtained by the proposed method and those secured by the method advocated by the Food and Drug Administration under date of February 15, 1933, showed a good agreement between the two methods, but, in the opinion of the authors, the latter method is unnecessarily complicated, and the results are, in some cases, difficult to reproduce. Table 3 shows the comparative results secured by analyzing aliquot portions of the same solutions by the two methods.

Figures given in Tables 1, 2, and 3 indicate that washings made in the manner indicated contain all of the lead on the surface of the apple with the exception of a fairly constant amount equal to approximately

TABLE 2. LEAD RECOVERED FROM APPLE WASH SOLUTIONS

	Lead in wash solution grains per pound	Lead added grains per pound	Total lead recovered grains per pound
Wash solution A	0.010		
	0.010		
	0.010		
	0.010	0.004	0.014
	0.010	0.004	0.014
	0.010	0.008	0.0175
Wash solution B	0.010	0.008	0.018
	0.009		
	0.009		
	0.009	0.004	0.013
	0.009	0.006	0.015
	0.009	0.010	0.019

TABLE 3. AMOUNTS OF LEAD FOUND IN SOLUTIONS OF APPLE WASHINGS BY THE PROPOSED METHOD AND BY THE METHOD OF THE UNITED STATES FOOD AND DRUG ADMINISTRATION

Sample No.	Lead by proposed method grains per pound	Lead by F. & D. A. method grains per pound
1	0.010	0.010
2	0.010	0.011
3	0.042	0.046
4	0.071	0.072
5	0.105	0.097

0.001 grain per pound of apples. Studies on the efficiency of the removal of arsenic by the wash solution have indicated that a similar fairly constant amount of approximately 0.001 grain of this element per pound of apples remains on the fruit. Apparently this remainder is independent of the amount on the fruit originally, and may represent that part of the spray residue which is firmly bound, either chemically or physically, to the surface of the apple. The figures in the tables also indicate that the proposed method allows the quantitative recovery of known amounts of lead under the same conditions as obtain in the actual determination, that is, in the presence of the waxes, carbohydrates, etc., that are normally present in the wash solution.

The accuracy of the method, as judged by several hundred determinations on apples, and by the recovery tests cited above and others, appears to be ± 0.001 grain per pound of apples.

The main advantages of the method here presented are the ease and rapidity with which results of a high degree of accuracy may be obtained. By actual test, the lead content of a sample of apples was determined in duplicate within one and one-half hours after the sample was presented at the laboratory. In addition, the absence of any involved chemical treatment makes the method readily adapted for field use.

Summary

A rapid method for the determination of lead in spray residues on apples is presented, based on the use of the Photronic cell. Under the conditions in the tests here reported, the method has an accuracy of 0.001 grain of lead per pound of apples, and allows lead determinations to be made with great rapidity. It is suggested that it may be useful as a field method.

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Effect of Concentrations of Nicotine on Growth and Development

II. GROWTH AND DEVELOPMENT OF CHICKS AS INFLUENCED BY THE ADDITION OF GROUND TOBACCO TO THE RATION*

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HUNTER and Haley (1930) called attention to the fact that a relatively high degree of tolerance was manifested by young chicks when their ration was supplemented with varying quantities of ground tobacco, *Nicotiana rustica*, a strain which possessed a nicotine content equal to 5 percent of its dry weight. The results showed that there was no apparent depression of the growth weight where chicks were fed these rations for a considerable period of time.

It was decided to continue these investigations in order to compare the relative effects of both high and low nicotine strains of tobacco, and to observe the relation of tobacco feeding to the control of worm infestation of the intestinal tract of the experimental birds, especially as it applied to the control of large roundworms (*Ascaridia lineata*).

In all cases the same basal ration was used, namely:

Yellow corn meal	40 lbs.
Wheat bran	15 lbs.
Flour wheat middlings	15 lbs.
Alfalfa meal	10 lbs.
Ground oats	10 lbs.
Dried skim milk	10 lbs.
White fish meal	5 lbs.
Meat scrap 55%	5 lbs.
Salt (NaCl)	1 lbs.
Cod liver oil	1 lbs.

EXP. I.—ARTIFICIAL INFESTATION

For this experiment 200 day-old Single Comb White Leghorns were divided into five groups, and placed in battery brooders having wire mesh floors. Group A-1 received no tobacco. The other groups received varying quantities, as recorded in Table 1. The chicks received their ration as all mash and it was supplied *ad libitum*. One-half of the entire dosage of tobacco was given the first week, at the end of which they received the full amount and so continued throughout the feeding period.

Five weeks after the birds were placed on their respective rations, one-half of each group was treated with artificially incubated eggs of roundworms. These eggs were introduced into the gizzards by means of a catheter, and not less than 5,000 eggs per bird. The remainder were treated in like manner at the end of the sixth week.

At the end of the experiment the intestines of all the chicks were examined. Roundworms were apparent in a number of individuals (Table 1). Throughout this experiment the birds were weighed bimonthly. The weights are given in Figure 1.

The data in Table 1 indicate that ground tobacco, *Nicotiana rustica*, with a nicotine content of 5 percent, acts as a definite con-

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TABLE 1.—Feed consumption, mortality, and percentage of roundworm infestation of chicks with and without ground tobacco as a supplement (13-week period)

Group	Concentration of <i>Nicotiana rustica</i> in ration	Infestation with roundworms	Mortality	Average weight of feed consumed per bird
	percent	percent	percent	grams
A-1	0.0	71.0	5.0	3220
A-2	0.2	5.3	0.0	3243
A-3	0.4	0.0	2.5	3265
A-4	0.8	0.0	0.0	3168
A-5	1.2	0.0	0.0	3209

trol for large roundworms in the intestinal tract. The data in Figure 1 indicate a slow rate of growth in Group A-1, which may be attributed to the result of parasitic infestation, although, in the majority of cases, the infested birds showed not more than three roundworms per bird in the intestinal tract.

In order to study the relative effect of a high-nicotine strain of tobacco as compared to a low-nicotine strain, ground cigar-leaf tobacco, with a nicotine content equal to 0.86 percent of its dry weight, was compared with *Nicotiana rustica* with a 5 percent nicotine content. Quantities of each tobacco were taken in order that each ration carried the same concentration of nicotine. For this trial, nine groups each, consisting of 25 day-old Single Comb White Leghorn chicks, were used. The same practices were followed as in the previous trials, the birds being kept free of roundworm infesta-

tion by confining them to wire floors. Data as to the quantity of tobacco fed and the feed consumed are given in Table 2, while the data relative to growth are given in Figure 2.

The data in Table 2 indicate that the mash proved unsatisfactory to the birds in Groups B-8 and B-9. Their mortality was high. Those in Group 9 exhibited a diarrheal condition not apparent in the other groups. Figure 2 shows that the birds receiving low-nicotine tobacco did not compare favorably in growth with those receiving the high-nicotine tobacco, when the same nicotine levels were maintained.

EXP. II.—NORMAL INFESTATION

It was decided to raise chicks on ground known to be infested with roundworms and feed them *Nicotiana rustica* as a supplement to the ration, in order to effect a measure

TABLE 2.—Feed consumption, mortality and percentage of both high- and low-nicotine strains of tobacco in the ration (10-week period)

Group	Concentration of ground tobacco in the ration		Mortality	Average weight of feed consumed per bird
	High nicotine	Low nicotine		
	percent	percent	percent	grams
B-1	0.0		0.0	2186
B-2	0.2		8.0	2252
B-3	0.4		4.0	2373
B-4	0.8		0.0	2283
B-5	1.2		4.0	2314
B-6		1.16	12.0	2378
B-7		2.33	8.0	2401
B-8		4.65	32.0	1929
B-9		6.98	40.0	1559

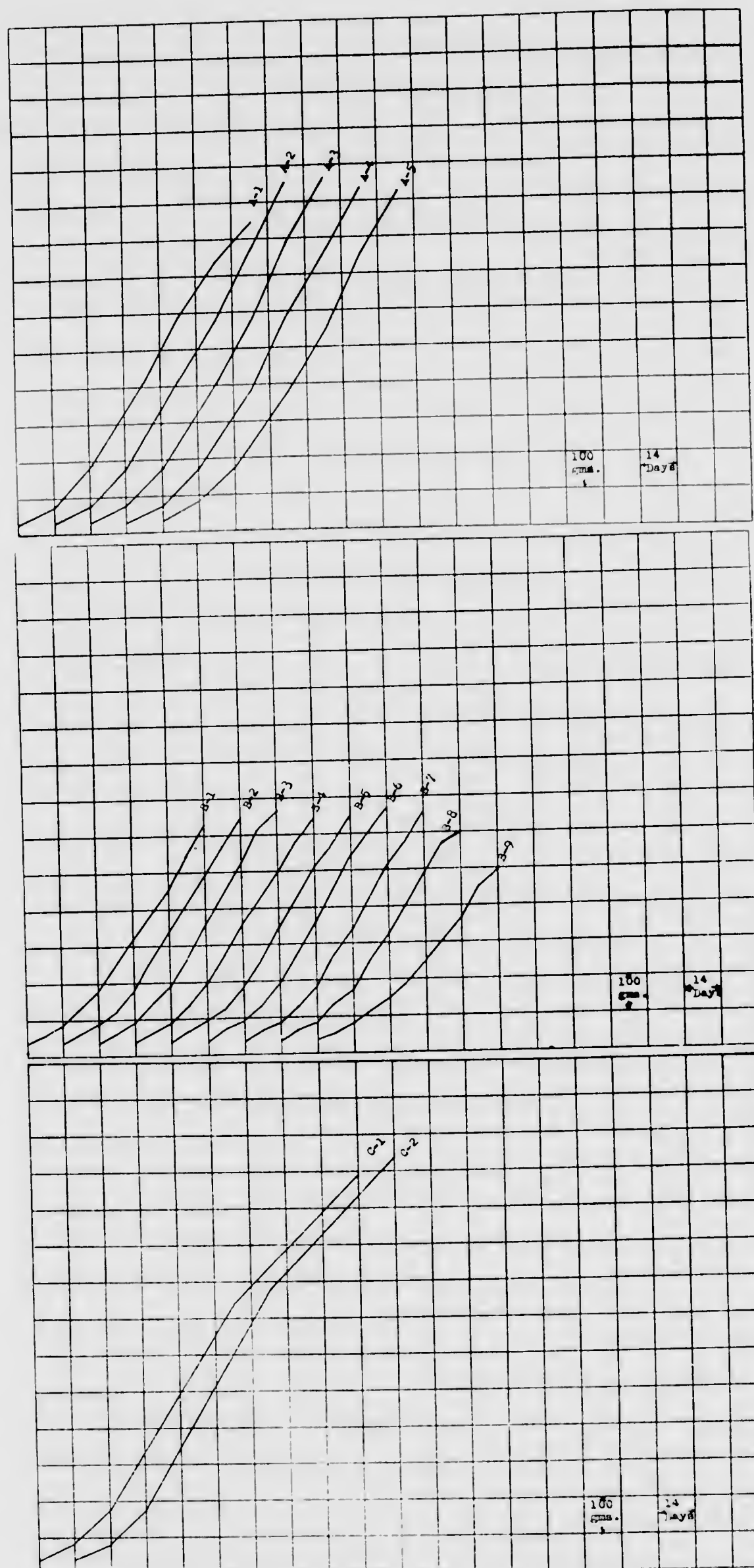

FIG. 1. (top)—Rate of growth as influenced by supplementary feeding of *Nicotiana rustica* (weighted averages of both sexes).

FIG. 2.(center)—Rate of growth as influenced by high and low-nicotine strains of tobacco (weighted averages of both sexes).

FIG. 3.—Rate of growth of chicks grown on infested ground as influenced by the supplementary feeding of *Nicotiana rustica* (weighted averages of both sexes).

TABLE 3.—Feed consumption, mortality, and worm infestation of chicks grown on infested ground (18-week period)

Group	Infestation with		Mortality	Average amount of feed consumed per bird
	<i>Ascaridia lineata</i>	<i>Heterakis gallinae</i>		
	percent	percent	percent	grams
C-1	14.0	61.0	25.8*	5380
C-2	1.0	56.0	20.8*	5190

* The high mortality experienced in this study was for the most part accidental, being caused by the birds piling when transferred from batteries to colony houses.

of control. Because of the limitations of physical equipment, it was impossible to carry through a series of group experiments. Hence it was decided to use a control group, and to administer to another group (C-2) a ration containing 0.4 percent of *Nicotiana rustica*. Each group contained 120 birds. They were kept in battery brooders for four weeks and afterward placed in colony houses for seven weeks. Finally they were transferred to the infested ground for seven weeks. The infestation, as shown in Table 3, was not high, even in the control group, probably because the soil was not heavily infested. However, this may have been due to the fact that they were quite mature when placed on the infested ground. According to Ackert (1930) the chances of producing artificial infestations of *Ascaridia lineata* in birds is slight after the birds have reached the age of 10-12 weeks.

Table 3 shows the feed consumption and percentage of worm infestation, while Figure 3 shows the data on growth.

CONCLUSIONS

These investigations have substantiated the findings of previous studies that the

feeding of ground *Nicotiana rustica*, a strain of tobacco possessing a nicotine content equal to 5 percent of its dry weight, at levels up to 1.2 percent of the ration does not interrupt the growth of chicks. On the other hand, the feeding of ground cigar-leaf tobacco with a nicotine content of 0.86 percent at levels of 4.65 percent or above retarded the growth of chicks and caused an increase in mortality.

The use of 0.4 percent of *Nicotiana rustica* was effective in the control of an artificial infestation of roundworms and appeared nearly, if not equally, effective in the control of a natural infestation. This level of *Nicotiana rustica* was not effective in the control of natural infestation of cecal worms (*Heterakis gallinae*).

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THE FOUNDATIONS OF NUTRITION. Mary Swartz Rose, Ph.D., Professor of Nutrition, Teachers' College, Columbia University. The Macmillan Co., New York City, 1933. Second Edition. xi + 630 pp. 101 Figs. 13 × 20 cm. \$3.00.

The author states that the "book is written for those who wish to live more intelligently. An effort has been made to present within a small space some of the fundamental principles of human nutrition in terms which call for no highly specialized training in those natural sciences upon which the science of nutrition rests."

That Dr. Rose has been successful in presenting the fundamentals of human nutrition in an interesting and effective manner is probably best indicated by the fact that Foundations of Nutrition has undergone many reprintings and one complete revision since it was first published in 1927.

The present book is rewritten and enlarged but follows the same general plan that characterized the first edition. A comparison of the first and second editions shows that the 12 chapters of the former have been expanded to 26 chapters in the latter. This has been done by the inclusion of outstanding researches published since the appearance of the first edition. Separate chapters are now devoted to each of the vitamins. The appendix, which consists of nine tables and one chart, has been revised and enlarged.

From the standpoints of binding, general appearance, size of pages, quality of illustrations and typography, the second edition appears to be a distinct improvement over the first edition.

The reviewer feels that the book is unique in that it discusses the fundamental principles of human nutrition in an elementary but authoritative and scientific manner and at the same time answers most of the important practical questions regarding the application of these principles to food selection and diet construction.

Dr. Rose's new text will undoubtedly continue to hold the unique position it has achieved for itself as the outstanding elementary textbook on human nutrition in the home economics field.

THE PENNSYLVANIA STATE COLLEGE
STATE COLLEGE, PA.

R. ADAMS DUTCHER

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7323 C

Effect of Type of Carbohydrate on Vitamins B and G Potency of Feces Voided by Rats.*

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State College.*

In a previous publication from this laboratory¹ the danger of coprophagy, as it affects the assay of vitamins B and G, was discussed. Similar observations had been reported by Roscoe.² More recently Booher and Kaneko³ have submitted data which led them to conclude that their assays for vitamin B (B₁) were not vitiated by coprophagy. The last mentioned investigators used raw corn starch as the source of carbohydrate. Since Roscoe had reported that she was unable to obtain characteristic growth responses from coprophagous rats when raw corn starch served as the source of carbohydrate, it seemed highly probable that the type of carbohydrate might be an important factor worthy of further study. This possibility has been further suggested by data obtained in this laboratory during a somewhat related investigation in which it was found that the quantity of vitamins B and G required to produce a unit increment of growth was considerably greater when sucrose was fed as the source of carbohydrate than it was when dextrinized corn starch was used.

The experiments here described were initiated to determine the extent to which growth responses in coprophagous rats could be affected by substituting carbohydrates of various types in the usual

* Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station February 21, 1934, as Technical Paper No. 637.

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³ Booher, L. E., and Kaneko, T., *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 69.

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basal ration deficient in vitamins B and G. Among the carbohydrates under investigation at present are: corn starch, dextrinized corn starch, glucose, sucrose, and lactose. While the results to date are insufficient for a complete statement, the differences obtained with diets containing sucrose and corresponding diets containing dextrinized corn starch are sufficiently marked to justify a preliminary note at this time.

The experimental technique employed was similar to that reported elsewhere.¹ The feces voided were removed from the cages daily, placed in a separate feed container and returned to the cage of the animal which voided them or to that of another designated animal. The growth records of a few typical animals are given in Chart 1.

Animal 8904, weight 40 gm., received the sucrose-containing diet (353), unsupplemented. This animal made about the same increase in weight during the first week of the experiment that is usually observed when young animals receive this diet. During the remainder of the experimental period, there was a consistent loss in weight. The animal manifested its first paralytic symptoms on the 26th day and died on the 31st day.

Animal 8894, weight 43 gm., also received the sucrose-containing diet unsupplemented. The increase in body weight during the first week (8 gm.) was somewhat greater than that usually obtained while using this diet. During the following 2 weeks there was a gradual loss in weight. Beginning on the 22nd day, the feces of this animal were collected daily, placed in a separate food-container and returned to the cage. These feces were consumed quite readily during the first few days, after which the animal refused to ingest appreciable amounts. In the meantime the animal continued to lose weight and manifested slight paralytic symptoms. Starting on the 52nd day and continuing through the 66th day, this animal was given one-half of the feces voided daily by its litter mate (animal No. 8893), which at this time was receiving a dextrin-containing diet (349). Animal 8894 consumed these feces very greedily. During this 14-day period, the paralytic symptoms disappeared and the animal increased 12 gm. in weight. On the 66th day feces feeding was discontinued and the animal lost weight gradually and died on the 81st day.

Animal 8902, weight 40 gm., received the dextrin-containing diet unsupplemented throughout the experimental period. During the first 4 weeks its deportment was quite comparable to that which is usually obtained on this diet. There were some indications, how-

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ever, that during the last 4 weeks this animal was practicing a slight degree of coprophagy. This condition seems to be extremely difficult to avoid when the diet contains a relatively high percentage of dextrinized corn starch, especially when the experimental animal has been over-depleted of its vitamin B stores. This animal was removed from the experiment at the end of the eighth week.

Animal 8893 (litter mate of 8894), weight 40 gm., received the

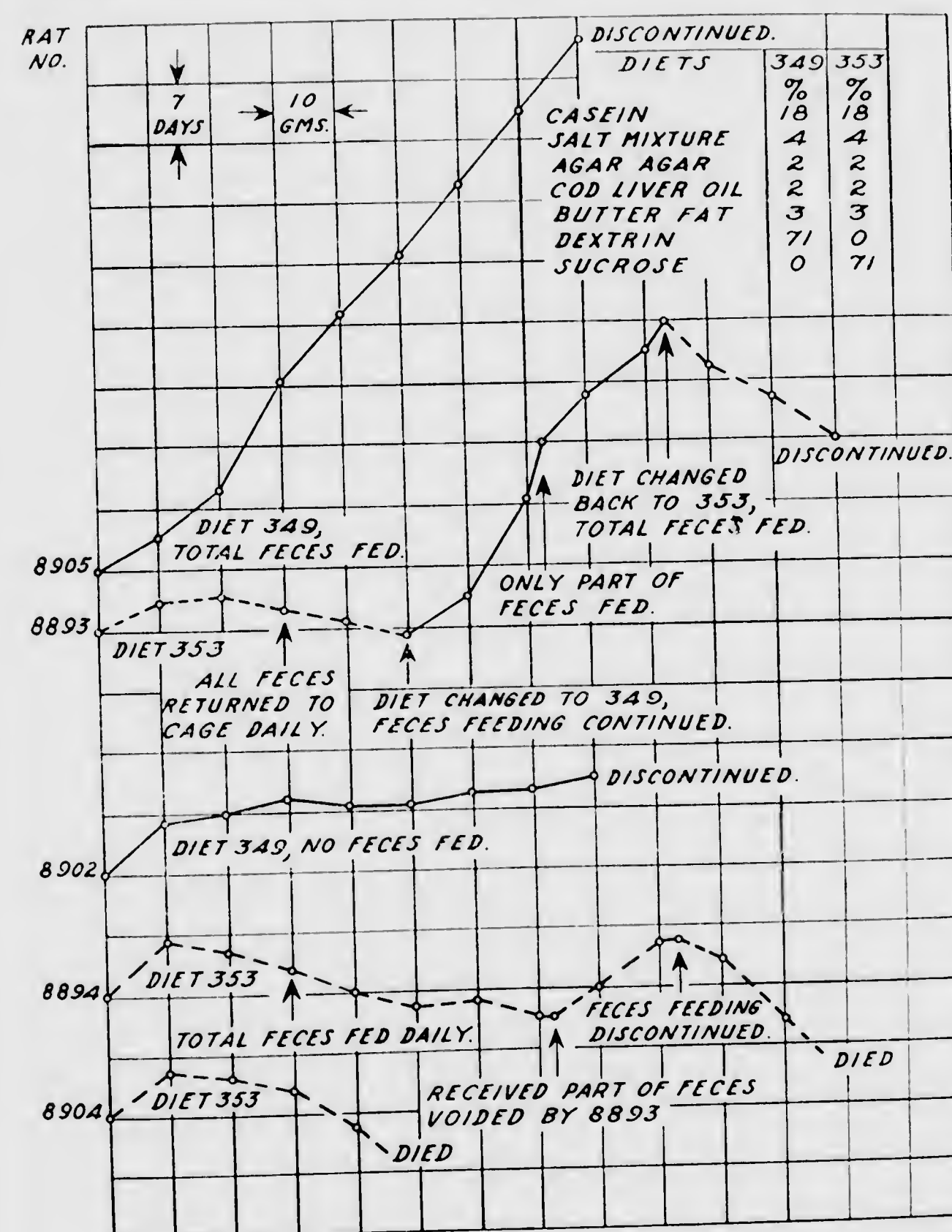


CHART I.

Showing typical growth responses made by young rats when fed vitamin B-complex deficient diets, unsupplemented and supplemented with feces voided by animals receiving the respective diets. The 2 diets differed in composition only in respect to the type of carbohydrate used.

RELATION OF CARBOHYDRATES TO COPROPHAGY

sucrose-containing diet (353), unsupplemented, during the first 3 weeks, and made a very typical growth response during this time. Starting with the 21st day, all feces voided by this animal were returned to the cage daily. From the 21st to the 35th day the animal continued to lose weight. On the 35th day the diet was changed from the sucrose diet to the dextrin diet, and the feces feeding was continued. During the next 17 days the weight of the animal increased 32 gm. Starting with the 52nd day and continuing for 2 weeks, only one-half of the feces voided by this animal was returned to the cage, the other half of the feces being fed to animal 8894 during this period. This animal (8893) continued to increase in weight so long as it received the dextrinized corn starch diet supplemented by its own feces. On the 66th day the diet was changed back to diet 353, and the feeding of all feces was continued from this date. Eighteen days later, the animal was removed from the experiment, after it had lost 19 gm. in weight.

Animal 8905 (litter mate of 8902 and 8904), weight 42 gm., received diet 349 throughout the feeding period. Starting with the second day of the experiment, all of the feces were returned to the cage daily. At first there was some hesitancy on the part of the animal to consume the entire supply of feces but this lack of appetite had completely disappeared by the fifteenth day. The animal made a total gain of 86 gm. during the 8 weeks' observation, and at the end of this time appeared to be normal in all respects.

Summary. Data are presented which indicate that the type of carbohydrate used in vitamin B and G deficient diets is an important factor in determining whether or not coprophagy will vitiate results when accurate assays of vitamins B and G are desired. Coprophagy appears to be of little consequence when sucrose is used as the sole source of carbohydrate, while dextrinized corn starch offers the possibility of introducing serious errors and incorrect conclusions if rats resort to coprophagy when this carbohydrate is used.

Experiments are now in progress in which an attempt is being made to find an explanation for the results described, and the study is being extended to include other types of carbohydrates.

A COMPARISON OF METHODS FOR THE DETERMINATION OF URIC ACID IN HUMAN, BOVINE, AND AVIAN BLOODS

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A COMPARISON OF METHODS FOR THE DETERMINATION OF URIC
ACID IN HUMAN, BOVINE, AND AVIAN BLOODS*†

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NUMEROUS methods for the determination of uric acid in human blood have been developed, the chief motive being to incorporate the higher degree of accuracy of the isolation methods with the greater simplicity and ease of manipulation of the direct methods. While several of these methods have given satisfactory results on human blood, few of them have been applied to the blood of other animals. Since in this laboratory we have been especially interested in the analysis of bovine and avian (chicken) bloods, the present study was undertaken to ascertain the most satisfactory method for the determination of uric acid in these bloods.

DISCUSSION OF DATA

In Table I data are presented to show the results of analyses of human, bovine, and avian bloods, using the Folin-Wu laked blood filtrate¹⁵ and the Folin unlaked blood filtrate.¹² The methods of Folin^{11, 13} of Folin as modified by Bulmer, Eagles and Hunter,⁸ of Benedict,² and of Brown⁷ were used with each of these filtrates, each method being applied to the filtrate directly and to the uric acid isolated as the silver salt from each filtrate. Thus twelve methods were used with each sample of blood.

The data in Table I show that in most cases the isolation methods give lower results than the direct methods. This fact is more evident in the case of laked than in the case of unlaked blood filtrates, the difference being especially pronounced in the case of laked filtrates from bovine blood. Direct and

*From the Department of Agricultural and Biological Chemistry of The Pennsylvania State College, State College of Pennsylvania.

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TABLE I
SHOWING A COMPARISON OF THE URIC ACID CONTENT OF HUMAN, BOVINE, AND AVIAN BLOODS BY THE METHODS OF FOLIN, BENEDICT, AND BROWN
ON LAKED AND UNLAKED BLOOD FILTRATES

SPL. NO.	LAKED BLOOD						UNLAKED BLOOD					
	FOLIN			BENEDICT			BROWN			FOLIN		
	DIRECT	ISOLATION*	DIRECT	DIRECT	ISOLATION	DIRECT	DIRECT	ISOLATION	DIRECT	DIRECT	ISOLATION*	DIRECT
MG. URIC ACID PER 100 C.C. OF BLOOD												
HUMAN												
1	4.2	3.2	3.4	3.4	2.9	3.7	3.1	2.4	2.8	2.8	3.5	3.2
2	5.2	4.1	4.5	4.5	4.5	5.6	5.4	4.1	4.5	3.8	5.9	4.6
3	4.6	3.3	3.6	3.6	3.7	3.5	3.4	3.5	2.7	2.6	5.2	4.1
4	5.7	4.9	4.8	4.8	4.5	4.5	4.6	2.8	4.6	3.3	5.3	3.9
5	5.3	4.6	4.2	4.2	4.7	5.4	3.3	4.6	4.1	4.7	5.8	5.8
6	3.9	2.8	3.1	3.1	3.6	4.6	3.3	3.2	3.2	3.2	4.3	4.2
7	4.3	3.4	3.6	3.6	3.7	4.6	3.4	3.4	2.9	3.4	3.8	4.0
Average	4.74	3.76	3.89	3.92	3.86	4.56	3.94	3.42	3.58	3.69	4.83	4.13
BOVINE												
1	2.2	1.5	1.4	1.4	0.7	2.0	0.5	1.3	1.4	0.3	1.2	no color
2	2.5	0.7	2.6	2.6	0.6	1.4	0.6	0.3	0.7	0.4	0.9	no color
3	2.5	0.6	2.2	2.2	0.9	1.4	0.7	0.4	0.7	0.7	0.6	no color
4	2.7	0.7	2.0	2.0	0.6	1.8	0.5	0.5	0.8	0.5	0.6	no color
5	2.2	0.7	2.0	2.0	0.6	1.7	0.8	0.5	0.9	0.5	0.6	no color
6	1.9	0.9	1.9	1.9	0.7	1.7	0.5	0.4	0.7	0.6	0.6	no color
Average	2.33	0.85	2.01	2.01	0.65	1.67	0.60	0.57	0.87	0.50	0.75	no color
AVIAN												
1	4.6	3.0	4.1	4.1	lost	4.4	4.1	3.2	3.8	3.0	4.3	lost
2	4.6	3.5	4.4	4.4	4.1	5.8	5.0	3.5	4.8	3.6	5.3	4.6
3	5.2	3.4	4.8	4.8	3.4	5.5	4.5	4.1	5.1	4.1	5.9	3.9
4	3.9	2.1	3.7	3.7	2.0	4.6	3.0	2.6	3.1	2.9	3.5	2.7
5	4.1	3.5	4.2	4.2	3.2	4.8	3.9	3.1	3.9	lost	5.2	4.9
6	3.9	2.3	3.9	3.9	4.9	4.6	3.1	2.2	3.1	2.2	4.8	3.2
7	5.1	3.5	5.4	5.4	8.1	8.1	7.0	6.0	5.4	5.6	8.0	7.5
8	5.5	3.3	4.5	4.5	5.0	6.1	3.4	4.1	4.3	3.8	5.8	4.0
9	3.1	2.6	3.9	3.9	2.6	4.3	3.0	2.6	4.2	3.4	4.7	3.3
10	2.6	1.8	2.8	2.8	1.7	4.1	2.0	1.6	2.6	1.9	3.2	2.1
11	4.3	4.0	5.1	5.1	3.9	6.1	4.3	3.0	4.9	3.7	5.1	4.5
Average	4.08	3.18	4.25	4.25	3.09	5.31	3.93	3.27	4.11	3.42	5.07	4.07

*Five cubic centimeters of silver lactate solution were used in these cases as suggested by Bulmer, Eagles, and Hunter.

isolation procedures agree more closely on unlaked than on laked filtrates. All isolation procedures give results which agree fairly well.

With regard to the great differences in uric acid values obtained by the direct and isolation procedures on bovine blood, the work of Benedict and his coworkers^{9, 10, 17} should be mentioned. They have isolated from the erythrocytes of bovine blood a combined form of uric acid which is not precipitated by silver-magnesia mixture. The low values obtained with the isolation method and with the direct methods on unlaked filtrates from bovine blood might be explained on this basis, since combined uric acid would not be precipitated by the silver lactate in the isolation procedure and, being in the corpuscles, would not be present in unlaked blood filtrates.

Bulmer, Eagles, and Hunter^{8, 16} ascribed the discrepancy between the direct and isolation methods to a substance "X" which they isolated from animal bloods, including bovine blood, and which they called a simple pyrimidine nucleoside. This compound is precipitated by silver lactate but is not liberated by acid sodium chloride, so that it would not appear in the isolation procedure.

Almost simultaneously, Benedict and his coworkers^{1, 3, 4} isolated from pig and human bloods an interfering substance "thiasine" or thioneine, as it was later called. They claimed that this compound is not the same as substance "X," and that it causes high values in the direct method.

From the averages given in Table I it appears that for human blood Benedict's direct method applied to Folin's unlaked blood filtrate gives results more closely agreeing with the isolation procedure than any of the other methods. In the case of bovine blood, Folin's direct method applied to an unlaked blood filtrate gives results agreeing most closely with the isolation procedure. In the case of avian blood, the agreement between the direct and isolation procedures on an unlaked filtrate is about the same for Folin's as for Benedict's method.

Brown's method, with one exception, gives higher results on human and avian bloods than the other methods. In the case of bovine blood, there was no color produced when Brown's isolation procedure was applied to the unlaked filtrate. When Brown's direct method was applied to a laked blood filtrate, the results were lower than the direct methods of Folin and of Benedict.

In 1931 Benedict⁵ suggested the use of tungstomolybdic acid for the preparation of laked blood filtrates. He⁶ also suggested a modification of the isolation procedure. In Table II Benedict's laked blood filtrate is compared with Folin's laked and unlaked filtrates from human, bovine, and avian bloods. Folin's direct¹³ and isolation⁸ methods are applied to all three filtrates, and Benedict's direct² and new isolation⁶ procedures are used on Benedict's laked filtrates.

From the averages in Table II it appears that in the case of human blood results by all the isolation methods agree closely. Of the direct methods that of Folin, used with his unlaked filtrate, gives results which agree most closely with the results obtained by the isolation procedure. It should be noted, however, that in Table I, with an unlaked filtrate, results by Benedict's direct method agreed more closely with the isolation procedure than did Folin's.

TABLE II
SHOWING A COMPARISON OF THE URIC ACID CONTENT OF HUMAN, BOVINE, AND AVIAN BLOODS
BY THE DIRECT AND ISOLATION METHODS OF FOLIN AND BENEDICT ON UNLAKED
AND TWO TYPES OF LAKED BLOOD FILTRATES

SPLE. NO.	LAKED-FOLIN		LAKED-BLOOD-BENEDICT				UNLAKED-FOLIN	
	FOLIN		BENEDICT		FOLIN		FOLIN	
	DIRECT	ISOLATION*	DIRECT	NEW	DIRECT	ISOLATION*	DIRECT	ISOLATION*
MG. URIC ACID PER 100 C.C. OF BLOOD								
HUMAN								
11	3.0	2.7	3.3	2.4			3.0	2.5
12	3.2	2.5	3.1	2.5			2.5	2.7
13	2.8	2.8	2.9	2.0			2.5	2.4
14	3.9	3.2	2.7	3.5			3.1	2.9
15	2.8	2.2	2.8	2.3	2.9	2.3	2.9	2.1
16	3.6	3.5	3.3	3.2	3.7	3.5	3.6	3.5
17	2.8	2.5	3.4	2.4	3.5	2.5	2.4	2.1
Average	3.16	2.77	3.07	2.61	3.36	2.76	2.86	2.60
BOVINE								
11	1.6	0.4	1.6	0.9	1.6	0.5	0.5	0.2
12	1.3	0.4	1.6	0.9	1.5	0.5	0.4	0.2
13	1.0	0.5	1.2	0.5	1.3	0.6	0.4	0.2
14	1.9	0.7	2.0	1.0	1.8	0.7	0.6	0.5
15	1.4	0.5	1.6	0.8	1.5	0.5	0.4	0.3
16	1.5	0.6	1.6	1.0	1.7	0.7	0.5	0.4
Average	1.45	0.51	1.60	0.85	1.57	0.58	0.47	0.30
AVIAN								
11	2.9	1.8	4.2	2.9	3.2	1.8	2.0	1.7
12	2.9	2.1	3.8	3.0	3.2	1.9	2.4	2.1
13	3.3	2.3	4.9	2.7	3.7	2.3	2.5	2.3
14	3.3	2.3	4.4	2.7	3.4	2.3	2.8	2.3
15	3.7	2.7	5.3	2.9	4.0	2.9	2.8	2.9
16	3.5	2.5	4.6	2.7	3.7	2.6	2.6	2.3
Average	3.27	2.28	4.55	2.81	3.53	2.30	2.51	2.27

All figures are averages of duplicate determinations.

*Five cubic centimeters of silver lactate solution were used in these cases as suggested by Bulmer, Eagles, and Hunter.⁸

With bovine blood results by the isolation method do not agree so closely as they do with human blood. Benedict's new method gives the highest values, while Folin's method, applied to his unlaked filtrate, gives the lowest values. In Table I Benedict's isolation procedure applied to Folin's unlaked blood filtrate gives lower results than Folin's isolation procedure.

In the case of avian blood, Benedict's new method gives higher results than any of the other procedures. Folin's method with his unlaked filtrate gives the closest agreement between direct and isolation procedures although in Table I the agreement is nearly as close when Benedict's procedure is used with Folin's unlaked filtrate.

In conclusion, it may be stated that for human and avian bloods, it makes little difference which method is used, provided an isolation procedure is followed. Of the direct methods those of Benedict and of Folin, with Folin's unlaked filtrate, give results agreeing closely with the isolation procedures. For bovine blood Folin's direct method, applied to his unlaked filtrate, agrees most closely with the isolation procedure. The authors have found Folin's method more satisfactory than Benedict's because the use of the urea-cyanide solution eliminates the formation of a troublesome precipitate which is always imminent when Benedict's method is used.

Since this paper was written, Folin¹⁴ has made several revisions in his direct method and in the preparation of his uric acid reagent. While lack of time prevents the repetition of the work here presented, it is felt that Folin's latest technic should be considered in selecting a method for the determination of uric acid in animal bloods.

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THE DISTRIBUTION AND CONDITION OF NITROGEN
IN THREE HORIZONS OF A DIFFERENTIALLY FERTILIZED
HAGERSTOWN CLAY LOAM SOIL PLANTED TO
APPLE TREES IN METAL CYLINDERS

BY

WALTER THOMAS

(Contribution from Pennsylvania Agricultural Experiment Station)

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THE DISTRIBUTION AND CONDITION OF NITROGEN IN THREE HORIZONS OF A DIFFERENTIALLY FERTIL- IZED HAGERSTOWN CLAY LOAM SOIL PLANTED TO APPLE TREES IN METAL CYLINDERS¹

By WALTER THOMAS

Professor of phytochemistry, Pennsylvania Agricultural Experiment Station

INTRODUCTION

In a recent paper (15)² the writer reported the utilization and recovery of nitrogen, phosphorus, and potassium by apple trees grown in metal cylinders for a period of 6½ years. These trees received (each spring) during the last 3 years of growth different combinations of the pure salts sodium nitrate, monocalcium phosphate, and potassium sulphate. It was shown that the ratio in which nitrogen (as N), phosphorus (as P₂O₅), and potassium (as K₂O) were absorbed from the added salts (NaNO₃, CaH₄(PO₄)₂, and K₂SO₄) by those trees optimum with respect to growth and reproduction, that is, the trees receiving the NPK and NP treatments, was 3:0.3:1.5 as compared with a 3:8:4 ratio actually applied. This latter ratio was based on the current orchard practice. The great divergence between these ratios indicated the need for information as to the condition of the added nitrogen, phosphorus, and potassium in the soil in order to determine the extent to which these "theoretical" quantities and ratios should be modified as a result of the changes produced by the interaction of the added salts with the soil. The present investigation reports the status of the nitrogen in the three soil horizons in the cylinders from which the trees referred to above were removed.

METHODS OF EXPERIMENTATION

Inasmuch as the detailed plan of this experiment has been reported elsewhere (3, 14, 16) a brief outline only is necessary.

The soil, the analysis of which has been recorded (11), was formed in place by the weathering of limestone to the lower Silurian formations, and is of Trenton origin. The excavation was made on a strip of land 110 by 11 feet adjacent to the college experimental orchard. The history of this land indicates that except for the droppings of cattle no dressings of fertilizer had ever been applied. It may, therefore, be described as a virgin forest soil. The mechanical analysis (11) suggests that the surface soil consists of a heavy silt loam underlain by a clay loam which becomes heavier in texture as the depth increases.

The soil was excavated from this strip by a scoop shovel. The layers from each of three horizons, viz, surface (0 to 7 inches), sub-surface (7 to 21 inches), and subsoil (21 to 53 inches), were kept separate and each was thoroughly mixed and weighed. The total

¹ Received for publication Nov. 4, 1933; issued July 1934. Technical Paper no. 613 of the Pennsylvania Agricultural Experiment Station. Presented before the American Society for Horticultural Science at the Boston (1933-34) meeting of the American Association for the Advancement of Science.
² Reference is made by number (italic) to Literature Cited, p. 856.

weight of the horizons was 54,180, 109,200, and 249,000 pounds, respectively. Inasmuch as there were 42 cylinders, an equal distribution (by weight) of each layer among the cylinders would require the following quantities of soil to be added to each of the cylinders: Subsoil, 5,930 pounds; subsurface soil, 2,600 pounds; and surface soil, 1,290 pounds. This equal distribution was effected by ascertaining the weight of each of the respective horizons required to fill a steel wheelbarrow similar to those used in highway construction work. Such wheelbarrows were used in filling the cylinders. Following the addition of each wheelbarrow load, uniformity in density of the soil was secured by means of heavy wooden mallets fitted with 3-inch cast-iron pipe handles 5 feet in length. The process of filling the cylinders was completed in the spring of 1920. Uniformity with respect to the nitrogen, phosphorus, and potassium content of the soil in the cylinders at this stage was established by analysis. The mean of 30 determinations for total nitrogen in the original soil is given in the first line of table 1. Any departure from these values greater than the error of analysis (± 0.0001 percent) must be attributed to causes resulting from differences in treatment.

The trees were planted in the spring of 1922. Up to the spring of 1924 the system of culture was similar in all cylinders. This consisted of green manuring with buckwheat and rye principally. In May 1924 half the cylinders were seeded with a mixture of Kentucky bluegrass, *Poa pratensis* L., and timothy, *Phleum pratense* L. These cylinders are designated "cylinders under sod." In the remaining half of the cylinders a tillage system was adopted. These latter cylinders are designated "cylinders under cultivation." A distinction must be noted with respect to the additions of nitrogen from 1925 until the end of the experiment in 1927. During these last 3 years of the experiment the cylinders under cultivation received 15.9 grams more nitrogen than the cylinders under sod. The reason for this is that it was then considered necessary to add equal amounts of organic matter to all the cylinders under cultivation. This was accomplished by growing rye outside the cylinders. For further details the paper by Anthony and Clarke (3, p. 251) should be consulted. All trees were allowed to grow without the addition of any mineral fertilizer until the spring of 1925, at which time differential treatment with different combinations of sodium nitrate, mono-calcium phosphate, and potassium sulphate was commenced. It is important to note that the conditions of this experiment preclude any erosion by water and practically none by wind.

The sodium nitrate was added according to the following schedule:

	Grams
April 18, 1925.....	906
May 3, 1926.....	45
June 7, 1926.....	453
June 20, 1926.....	408
May 5, 1927.....	337
May 18, 1927.....	338
June 10, 1927.....	337
Total.....	2,824

This total of 2,824 g of sodium nitrate is equivalent to 465.5 grams of elemental nitrogen.

During the period from September 20 to 28, 1927, the trees were dug up and soil samples representative of the three horizons were taken, by the method of successive quartering, from each of the cylinders from which the trees had been removed. These samples were dried at 75° C. and then sieved through a 1-millimeter sieve (4) and stored in glass jars in the dark. Analyses of the trees have already been reported (14).

Total nitrogen was determined by the Kjeldahl-Gunning method to include the nitrogen of nitrates, a 15-g charge being used for the surface soil and 30-g for the subsurface and subsoil (4). Nitric nitrogen was determined by the Devarda alloy method, a 200-g charge being used (1). The analytical data in the tables are the means of closely agreeing triplicate determinations. The quantities of ammonia nitrogen and nitrous nitrogen in all horizons were insignificant. All calculations are made on a moisture-free basis.

EXPERIMENTAL DATA

Table 1 gives the percentage and absolute amounts of total nitrogen in each of the three soil horizons; that is, the 0 to 7 inch, the 7 to 21 inch, and the 21 to 53 inch.

TABLE 1.—Percentages and absolute amounts of total nitrogen in the respective horizons before the trees were planted and at the end of the experiment

Treatment	Total nitrogen			Absolute amount total nitrogen			
	Surface (0-7 inches)	Subsur- face (7-21 inches)	Subsoil (21-53 inches)	Surface (0-7 inches)	Subsur- face (7-21 inches)	Subsoil (21-53 inches)	Total (0-53 inches)
Soil before trees were planted.....	Percent 0.08650	Percent 0.05003	Percent 0.03523	Grams 506.1	Grams 589.7	Grams 946.8	Grams 2,042.6
Sod:							
Check.....	.08219	.04600	.03400	477.9	542.5	914.5	1,934.9
PK.....	.08200	.04700	.03498	479.8	554.3	941.4	1,975.5
NPK.....	.08200	.05160	.03900	478.6	613.2	1,049.0	2,140.8
NP.....	.08180	.05250	.03900	478.6	619.1	1,049.0	2,146.7
NK.....	.08200	.05500	.04040	479.8	648.6	1,083.0	2,211.4
N.....	.08200	.05200	.04000	479.8	625.0	1,075.9	2,180.7
Cultivation:							
Check.....	.07110	.05100	.03445	415.9	601.5	926.6	1,944.0
PK.....	.07485	.05000	.03571	436.4	590.4	960.5	1,987.3
NPK.....	.07800	.05290	.04310	456.4	620.8	1,146.6	2,223.8
NP.....	.07900	.05230	.04250	462.2	614.4	1,142.9	2,219.5
NK.....	.07820	.05500	.04350	457.6	648.8	1,170.1	2,276.5
N.....	.07890	.05200	.04200	461.6	620.2	1,131.9	2,213.7

Table 2 gives the percentage and absolute amounts of nitric nitrogen and of nonnitric nitrogen. The latter values were obtained by difference between the total nitrogen and the nitric nitrogen.

TABLE 2.—Percentage and absolute amounts of nitric and nonnitric nitrogen in the respective horizons before trees were planted and at the end of the experiment

Treatment	Nitric nitrogen			Absolute amount nitric nitrogen			
	Surface (0-7 inches)	Subsurface (7-21 inches)	Subsoil (21-53 inches)	Surface (0-7 inches)	Subsurface (7-21 inches)	Subsoil (21-53 inches)	Total (0-53 inches)
	Percent	Percent	Percent	Grams	Grams	Grams	Grams
Soil before trees were planted...	0.00170	0.00053	0.00023	9.9	6.3	6.2	22.4
Sod:							
Check	.00069	.00080	.00060	4.0	9.4	16.1	29.5
PK	.00070	.00100	.00055	4.1	11.8	17.7	33.6
NPK	.00220	.00760	.00150	12.9	89.6	121.0	223.5
NP	.00200	.00720	.00500	11.7	84.9	133.5	230.1
NK	.00290	.00600	.00560	16.9	106.1	147.0	270.0
N	.00240	.00820	.00550	14.0	96.7	147.9	258.6
Cultivation:							
Check	.00110	.00100	.00065	6.4	11.8	17.5	35.7
PK	.00085	.00120	.00071	3.5	14.1	19.1	36.7
NPK	.00210	.00940	.00110	12.3	72.4	111.0	195.7
NP	.00200	.00610	.00460	11.7	69.6	123.5	204.8
NK	.00270	.00690	.00590	15.8	81.5	158.7	256.0
N	.00290	.00760	.00500	16.9	89.5	136.7	243.1
Treatment	Nonnitric nitrogen			Absolute amount nonnitric nitrogen			
	Surface (0-7 inches)	Subsurface (7-21 inches)	Subsoil (21-53 inches)	Surface (0-7 inches)	Subsurface (7-21 inches)	Subsoil (21-53 inches)	Total (0-53 inches)
	Percent	Percent	Percent	Grams	Grams	Grams	Grams
Soil before trees were planted...	0.0848	0.0495	0.0350	496.2	583.4	940.6	2,020.2
Sod:							
Check	.0815	.0452	.0334	473.9	533.1	898.4	1,905.4
PK	.0813	.0460	.0343	475.7	542.5	923.7	1,941.9
NPK	.0798	.0444	.0345	468.9	523.6	928.0	1,915.5
NP	.0798	.0453	.0340	466.9	534.2	915.5	1,916.6
NK	.0791	.0460	.0348	462.9	542.5	936.0	1,941.4
N	.0796	.0448	.0345	465.8	528.3	928.0	1,922.1
Cultivation:							
Check	.0700	.0500	.0338	409.5	589.7	909.1	1,908.3
PK	.0740	.0488	.0350	432.9	576.2	941.4	1,950.5
NPK	.0759	.0465	.0390	444.1	548.4	1,035.6	2,028.1
NP	.0770	.0462	.0379	450.5	544.8	1,019.4	2,014.7
NK	.0755	.0481	.0376	441.8	567.3	1,011.4	2,020.5
N	.0760	.0450	.0370	444.7	530.7	995.2	1,970.6

DISCUSSION OF DATA

TOTAL NITROGEN

The data in table 1 indicate that at the end of the experiment the total nitrogen content of the surface soil in all cylinders under sod was slightly greater than that in cylinders under cultivation, although, as has already been pointed out, the cylinders under cultivation had received 15.9 grams more nitrogen than the corresponding cylinders under sod. The differences between the total nitrogen content of corresponding cylinders under the two systems in the respective horizons are shown in table 3.

The data in table 3 do not take into account the nitrogen removed by the trees and, in sod, by the grass also. The disappearance of nitrogen (as total nitrogen) when the amounts removed by the trees are taken into account is shown in table 4. This disappearance is called by some investigators "the apparent loss of nitrogen."

TABLE 3.—Difference ^a (in grams) between the amounts of total nitrogen present in the 3 horizons of the 2 systems

Horizon	Check	PK	NPK	NP	NK	N
Surface (0-7 inches)	+62.0	+43.4	+23.4	+16.4	+22.2	+18.2
Subsurface (7-21 inches)	-59.0	-36.1	-7.6	-4.7	0	+4.8
Subsoil (21-53 inches)	-12.1	-19.1	-97.6	-93.9	-87.1	-56.0
Total (0-53 inches)	-9.1	-11.8	-81.8	-72.8	-64.9	-33.0

^a The sign indicates the amount in grams by which the total nitrogen under sod is greater than (+) or less than (-) under cultivation.

TABLE 4.—Nitrogen disappearance (grams) calculated on the total nitrogen of the soil in the whole layer (0 to 53 inches) at the end of the experiment

Item	Sod					
	Check	PK	NPK	NP	NK	N
(1) Amount N present in soil before experiment	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6
(2) Amount N added in NaNO ₃ (=465.0 g)+seeds (=2.5 g)	2.5	2.5	467.5	467.5	467.5	467.5
(3) Amount N from (1)+(2)	2,045.1	2,045.1	2,510.1	2,510.1	2,510.1	2,510.1
(4) Amount N found	1,934.9	1,975.5	2,142.0	2,146.7	2,211.4	2,180.7
(5) Loss of N from soil	-110.2	-69.6	-368.1	-363.4	-298.7	-329.4
(6) Total amount N absorbed by trees during growth (tops and roots)	36.5	56.8	201.4	190.4	133.4	124.3
(7) Disappearance of N by leaching and possibly as gaseous N during the 6½ years of the experiment	-73.7	-12.8	-166.7	-173.0	-165.3	-205.1

Item	Cultivation					
	Check	PK	NPK	NP	NK	N
(1) Amount N present in soil before experiment	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6
(2) Amount N added in NaNO ₃ (=465.0 g)+seeds (=2.5 g)	19.4	19.4	483.4	483.4	483.4	483.4
(3) Amount N from (1)+(2)	2,062.0	2,062.0	2,526.0	2,526.0	2,526.0	2,526.0
(4) Amount N found	1,944.0	1,987.3	2,223.8	2,219.5	2,276.5	2,213.7
(5) Loss of N from soil	-118.0	-74.7	-302.2	-306.5	-249.5	-312.3
(6) Total amount N absorbed by trees during growth (tops and roots)	53.5	63.4	180.9	170.3	131.8	121.3
(7) Disappearance of N by leaching and possibly as gaseous N during the 6½ years of the experiment	-64.5	-11.3	-121.3	-136.2	-117.7	-191.0

Considering the whole depth 0 to 53 inches, the losses from the nitrogen-treated cylinders are greater under sod than under cultivation. The amounts by which the losses (in grams) under the former system exceed those under the latter are: NPK, 45.4; NP, 36.8; NK, 47.6; and N, 14.1. These differences appear to be related to the accretion of nonnitric nitrogen in the subsoil of the cylinders under cultivation. The net result is a gain in nonnitric nitrogen when calculated on the whole depth (0 to 53 inches) in the treated cylinders under cultivation as compared with those under sod. This point is discussed later.

Line 7 of table 4 gives the losses by leaching and possibly as gaseous nitrogen (i.e., the so-called "nitrogen balance") for the whole soil layer (0 to 53 inches). The same data calculated for the surface 0 to 7 inches and subsurface 0 to 21 inches only are shown in table 5.

TABLE 5.—Nitrogen balance (in grams) calculated to less than full depth

Horizon	Check	PK	NPK	NP	NK	N
0 to 7 inches:						
Under sod	+5.8	+34.0	-292.4	-304.6	-360.4	-369.5
Under cultivation	-56.1	-25.7	-352.2	-357.0	-400.1	-406.6
0 to 21 inches:						
Under sod	-41.4	-7.4	-268.9	-275.2	-301.5	-334.2
Under cultivation	-44.3	-25.0	-321.1	-332.3	-341.0	-376.1

The nitrogen balance is seen to vary with the depth of soil upon which the calculations are based. In the 0- to 7-inch layer, a nitrogen gain is indicated in the cylinders under sod to which no mineral nitrogen was added. But if the calculations are based on the 0- to 21-inch layer or on the whole depth, 0- to 53-inch layer, losses of nitrogen are definitely established. The larger losses shown in the nitrated cylinders in the 0- to 7-inch and 0- to 21-inch layers as compared with the 0- to 53-inch layer appear to be only an expression of the fact that the quantity of nitrates (nitric nitrogen) becomes greater with depth.

More information with respect to the status of the nitrogen is obtained by considering the nitric and nonnitric fractions of the total nitrogen separately. These are shown in table 2.

NITRIC NITROGEN (NITRATES)

MOBILITY OF ADDED NITRIC NITROGEN

Table 2 shows that the nitric nitrogen calculated on a percentage basis, i.e., the concentration of nitrates, is greater in the subsurface than in either of the other horizons. The absolute amount of nitric nitrogen, however, is greater in the subsoil in all cases. The last application of NaNO_3 (167 grams nitrogen) was made in the spring of 1927, 4½ months before the trees were removed. Presumably, therefore, this increased concentration of nitric nitrogen in the subsurface is merely an expression of the movement of nitrogen as nitric nitrogen from this last application, and when taken in conjunction with the data for the nitric nitrogen in the check and PK cylinders, suggests that the greater part of the last application was still in the 7- to 21-inch layer at the conclusion of the experiment.

NITRIC NITROGEN UNDER SOD AND CULTIVATION

It will be recalled that each of the cylinders under cultivation received 15.9 grams more nitrogen in the form of rye cover crop than the cylinders under sod. Now the concentration of nitric nitrogen in the whole depth (0 to 53 inches) of the check cylinder under cultivation is 35.7 grams and that of the cylinder under sod is 29.5 grams as compared with 22.4 grams in the original soil. However, in the cylinders which received mineral nitrogen in addition to that introduced by the green manures (the NPK, NP, NK, and N cylinders) the concentration of nitric nitrogen in the whole depth is much greater in all cases in the cylinders under sod. This may be only another expression of the difference in the status of the soil nitrogen in the three horizons under sod and cultivation previously referred to in the discussion of the disappearance of nitrogen (as total N) by leaching and possibly as gaseous nitrogen. This will be brought out more clearly in the discussion of the nonnitric nitrogen fraction.

AN INVENTORY OF NITRIC NITROGEN

A more complete picture of the status of the nitrates may be obtained from the inventory of nitric nitrogen shown in table 6, in which account has been taken of the nitric nitrogen equivalent to that absorbed by the trees under the different treatments from the added NaNO_3 .

TABLE 6.—Inventory of nitric nitrogen (in grains) at end of experiment (0 to 53 inches)

Item	Sod					
	Check	PK	NPK	NP	NK	N
(1) N added to each cylinder in the form of NaNO_3	0	0	465.5	465.5	465.5	465.5
(2) N absorbed by each tree during growth and also (in sod) by the grass	36.5	56.8	201.4	190.4	133.4	124.3
(3) N absorbed by each tree from added NaNO_3	0	0	132.7	132.4	91.9	83.6
(4) Theoretical amount of N expected in soil	29.5	33.6	332.8	333.1	373.6	381.9
(5) N found			223.5	230.1	270.0	258.6
(6) Disappearance of nitric N during the 6½ years of the experiment			109.3	103.0	103.6	123.3

Item	Cultivation					
	Check	PK	NPK	NP	NK	N
(1) N added to each cylinder in the form of NaNO_3	0	0	465.5	465.5	465.5	465.5
(2) N absorbed by each tree during growth and also (in sod) by the grass	53.5	63.4	180.9	170.3	131.8	121.3
(3) N absorbed by each tree from added NaNO_3	0	0	117.5	93.2	78.3	67.8
(4) Theoretical amount of N expected in soil	35.7	37.0	348.0	372.3	387.2	397.7
(5) N found			195.7	204.8	256.0	243.1
(6) Disappearance of nitric N during the 6½ years of the experiment			152.3	167.5	131.2	154.6

The nitric nitrogen absorbed by the trees (table 6, line 3) was obtained in a manner similar to that described in an earlier paper (15, pp. 570-573). The values in line 3 represent the difference between the amount of nitrogen absorbed by a tree which received additions of another element (or other elements) than nitrogen and a tree from which nitrogen was omitted. The values so obtained may not be mathematically exact, inasmuch as the Wirkungskwert (effect factor) of an element may not be the same in the presence of another factor or factors as when that factor operates alone. Nevertheless, there are numerous experiments that lend support to Mitscherlich's contention (7) that the Wirkungskwert of an element may be fairly constant, especially under the controlled conditions of such an experiment as the present one. The method is believed to be sufficiently accurate to bring out more clearly any characteristic differences in the status of the nitric nitrogen of the respective treatments and especially with respect to differences between the two cultural systems. The procedure adopted may be more readily understood from the following algebraical analysis:

Let a = amount of nitrogen present in the soil of each cylinder before the trees were planted.

Let x = amount of nitrogen added to each cylinder in rain and snowfall.
 Let y = amount of nitrogen added to each cylinder under cultivation in the form of organic matter (buckwheat and rye).
 Let z = the total amount of sodium nitrate added to each of the "nitrated" cylinders.

Now the nitrogen-treated trees have obtained the nitrogen absorbed by them from all of the foregoing sources, and the trees which did not receive mineral nitrogen (NaNO_3) additions absorbed nitrogen from all of these sources except z .

For greater simplicity and clarity, let us first of all consider the absorption of nitrogen from only two of the trees on which chemical analyses were made; namely, the NPK and the PK trees, both under the tillage system.

The amount of nitrogen absorbed by the NPK tree during the whole period of its growth will be some fraction of $a+x+y+z$. Let this fraction be designated $k(a+x+y+z)$. Similarly, the amount of nitrogen absorbed by the PK tree during the whole period of its growth will be some fraction of $(a+x+y)$, which will be designated $k'(a+x+y)$.

$$\text{Let } \frac{1}{s} = k(a+x+y+z) \text{------(1)}$$

$$\text{Let } \frac{1}{r} = k'(a+x+y) \text{------(2)}$$

Then, by subtraction

$$\frac{1}{s} - \frac{1}{r} = k(a+x+y+z) - k'(a+x+y) \text{------(3)}$$

Now, if $k(a+x+y)$ is equal to or very nearly equal to $k'(a+x+y)$,

$$\text{then, from (3), } \frac{1}{s} - \frac{1}{r} = kz \text{------(4)}$$

or, expressed in words, the fraction of the nitrogen added to the NPK tree in the form of sodium nitrate is obtained by difference between the total amount of nitrogen absorbed by that tree and that absorbed by the PK tree.

In the present experiment the trees were grown without mineral salt additions for the first 4 years. The factor z then of equation (1) does not enter into the picture during this period.

For the purpose of the present analysis the difference between the quantities $k(a+x+y)$ must be very small as compared with the quantity kz . In further support of this contention may be cited the mathematical analysis given by the writer in an earlier paper (13).

The amounts of nitrogen applied as sodium nitrate were 149 g in 1925, 149 g in 1926, and 167 g in 1927, a total of 465 g. A comparison of these quantities of added nitrogen with the quantities actually present (table 6, line 5) shows that all of the "nitrated" cylinders contained at the end of the experiment more than enough nitrates to account for the amount (167 g) added the last year of the experiment, 4½ months before the samples were taken in the fall of 1927, and in addition a considerable portion of the nitrogen added in the second application in the spring of 1926. The total precipitation during the period between one application and the next was: May 18, 1925, to May 2, 1926, 31.8 inches; May 3, 1926,

to May 4, 1927, 44 inches; May 5, 1927, to September 20, 1927, 18.6 inches. In addition, 2 inches of artificial watering was applied in May 1926 and 1 inch in August 1927.

The Rothamsted experiments (9) on the losses of nitrogen in the drainage waters from a plot of arable land kept free of vegetation since 1870, which received no artificial additions of nitrogen, are frequently cited in support of the view that nitrates are readily leached from soils. At the end of 47 years the amounts of nitric nitrogen found in the drainage waters were equal to the total losses of nitrogen from the soil. The rate of loss was equal to 40 pounds per annum per acre in the earlier years and below 25 pounds per annum per acre in the later years.

In some soil types in Tennessee (8), however, when nitrogen was applied as sodium nitrate to lysimeters kept bare of vegetation, the leaching (outgo) of nitrogen in 2½ years was as low as 34 percent. Mooers and MacIntire attribute this relatively small loss to the influence of the heavy clay subsoil into which the nitrate ion passes as magnesium and calcium nitrates through base exchange.³

The investigations of De Sornay (10) also indicate that nitrates may remain in the soil available to plants for long periods, moving upward or downward according to moisture conditions. The upward capillary attraction was found to be much more rapid than the downward displacement by rain. De Sornay reports that Demolon and Brouet at Aisne found, in an uncropped, light, sandy garden soil, that after a rainfall of 9.8 inches during a period of 2 months more than one half of the added sodium nitrate remained in the first 8 inches.

More recently Ames (2) has reported that during the period 1928-30 the nitric nitrogen content of the soil under corn or soybeans never exceeded 50 pounds per acre in the surface 6½ inches, but in 1931, after a year of drought, the nitric nitrogen content reached 300 pounds per acre.

The problem is summed up by MacIntire⁴ as follows:

It is exceedingly difficult to make an unqualified statement as to the fate of added nitrogen. This will vary with the soil, alkalinity or acidity, climatic conditions, the amount of added nitrogen, absence or presence of growing plants and the type of these, and periodicity of rainfall, together with the very important fact of depth and type of subsoil.

In the present experiment the significant fact is that the disappearance of nitric nitrogen is greater in all the cylinders under cultivation to which mineral nitrogen was added than under the corresponding cylinders under sod.

NONNITRIC NITROGEN

The nonnitric nitrogen consists of (1) nitrogenous organic material potentially "available" but not yet decomposed; (2) the humus nitrogen characterized by marked stability; (3) the nitrogen synthesized by the micro-organisms; (4) ammonia nitrogen absorbed by the colloidal soil complex.

The apparent gain or loss in integral numbers with respect to the nonnitric nitrogen is given in table 7. The quantities of nitrogen shown in line 4, that is, the amount of nitrogen absorbed by the trees from sources other than the nitrogen added as NaNO_3 , were obtained

³ MACINTIRE, W. H. Private communication.

⁴ MACINTIRE, W. H. See footnote 3.

by using the quantities of nitrogen absorbed by corresponding treatments to which no nitrogen was added. For example, the amount of nitrogen absorbed by the NPK tree in sod from sources other than that equivalent to the added NaNO_3 was obtained by difference between this quantity and that absorbed by the PK tree also growing in sod. For reasons already given these values have no pretention to mathematical exactness. But in the present analysis they serve for all practical purposes to furnish a picture of the changes in non-nitric nitrogen of the original soil as a result of the various treatments under the two culture systems.

TABLE 7.—Gain or loss (grams) of nonnitric nitrogen from whole depth of soil (0 to 53 inches) by the end of the experiment

Item	Sod					
	Check	PK	NPK	NP	NK	N
(1) Nonnitric N before experiment.....	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2
(2) Nonnitric N at end of experiment.....	1,905.4	1,941.9	1,918.5	1,916.6	1,941.4	1,922.1
(3) Actual loss or gain by soil.....	-114.8	-78.3	-101.7	-103.6	-78.8	-98.1
(4) N absorbed per tree from sources other than the added NaNO_3	36.5	57.0	57.0	49.9	37.4	37.4
(5) Apparent total loss or gain during the 6½ years of the experiment.....	-78.3	-21.3	-44.7	-53.7	-41.4	-60.7

Item	Cultivation					
	Check	PK	NPK	NP	NK	N
(1) Nonnitric N before experiment.....	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2
(2) Nonnitric N at end of experiment.....	1,908.3	1,950.5	2,028.1	2,014.7	2,020.5	1,970.6
(3) Actual loss or gain by soil.....	-111.9	-99.7	+7.9	-5.5	+3.3	-49.6
(4) N absorbed per tree from sources other than the added NaNO_3	53.5	63.4	63.4	77.1	53.5	53.5
(5) Apparent total loss or gain during the 6½ years of the experiment.....	-58.4	-6.3	+71.3	+71.6	+53.8	+3.9

In both culture systems there is a disappearance of nonnitric nitrogen in the cylinders which received no mineral nitrogen additions (check and PK cylinders), the loss being greater under sod. But, whereas relatively large accretions of nonnitric nitrogen have occurred in the NPK, NP, NK, and N cylinders under cultivation, losses of nonnitric nitrogen have occurred from the corresponding cylinders under sod. These differences are much greater than can be accounted for by sampling or analytical errors. Although uniformity with respect to content of nitrogen, phosphorus, and potassium was established in the cylinders before the experiment began, there may have existed differences in respect to the physical condition that would preclude a definite and unqualified interpretation of the differences existing with respect to the condition of the nitrogen under sod and cultivation. Three explanations may be advanced:

(1) Assimilation of nitrogen added as NaNO_3 by micro-organisms. But the difficulty lies in explaining why assimilation should have occurred in the cylinders under cultivation and not in those under sod. Carbon dioxide accumulation under grass (12) may be the differential factor. Some nitrogen would appear to have been brought up and immobilized in the surface soil by the grass roots in the sod system, inasmuch as the quantity of nitrogen (as total and nonnitric nitrogen)

of the surface soil is higher in all cases under grass than under cultivation. But the results for the whole depth (0 to 53 inches) show that this explanation is insufficient to account for the entire difference in the amount of nitrogen in corresponding cylinders in the two systems.

(2) The peptization of nitrogenous organic material by NaNO_3 in the first horizon and subsequent leaching. Hardpan formation was particularly noticeable in the nitrogen-treated cylinders under cultivation. Cracks and fissures, therefore, may have assisted the downward movement.

(3) The greater root system under cultivation. Except in one tree (NPK) the root systems were larger in the trees grown under cultivation. The weights of the root systems are given in table 8.

TABLE 8.—Weights in grams of the respective root systems in soils in sod and under cultivation

Condition	Check	PK	NPK	NP	NK	N
Sod.....	9,950	8,325	13,040	12,730	11,090	10,366
Cultivation.....	10,870	10,695	12,395	13,075	11,700	12,275

The higher nitrogen content of the soil under cultivation may have arisen from sloughed-off portions of fibrous roots that might have been incorporated in the soil in the process of preparation for analysis.

The extensive literature of the problem of the mineralization of nitrogen in the soil has been reviewed by Lemoigne (5), Lyon (6), and Waksman (17). The results presented in this paper suggest the desirability of further investigation of the problem.

SUMMARY

The distribution of total nitrogen and also of the nitric and nonnitric fractions in three horizons, viz, 0 to 7 inches, 7 to 21 inches, and 21 to 53 inches, of a Hagerstown clay loam soil contained in cylinders planted to apple trees and treated with different combinations of sodium nitrate, monocalcium phosphate, and potassium sulphate are given in percentage and in absolute amounts.

In all treatments the total nitrogen of the surface soil under sod was somewhat greater than under cultivation. In the subsurface the differences in total nitrogen were small except in the check cylinders under sod, in which it was less than under tillage. In all treatments the total nitrogen of the subsoil was greater in the cylinders under cultivation than in the corresponding cylinders under sod. For the whole depth (0 to 53 inches), the total nitrogen at the end of the experiment was greater in all cylinders under cultivation than in those under sod.

The disappearance of nitrogen (as total nitrogen) by leaching, and possibly as gaseous nitrogen, was greater under sod than under cultivation in all cases.

The movement of nitric nitrogen is discussed. It is concluded that leaching of nitrates from this heavy soil was not very rapid.

The disappearance of nitric nitrogen, when account has been taken of the nitric nitrogen absorbed by the trees equivalent to that added as NaNO_3 , was greater in all nitrated cylinders under cultivation than

in corresponding cylinders under sod. This difference is accounted for by an accretion of nitrogen as nonnitric nitrogen in the subsoil under cultivation but not in that under sod.

Results with respect to nitrogen gains or losses based on the soil to a depth of 53 inches are very different from those based on the 0- to 7-inch or 0- to 21-inch depths.

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VITAMIN STUDIES XX. THE EFFECT OF VARIOUS METHODS OF PASTEURIZATION ON THE VITAMIN B AND THE VITAMIN G CONTENT OF COW'S MILK*

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With the recognition of vitamins B and G as separate entities, it has become necessary to re-evaluate much of the previous work regarding the thermostability of the vitamin B complex. Furthermore, the methods for the biological assay of vitamins B and G have been undergoing improvement and refinement with the result that many of the recent researches on the vitamin B and G content of cow's milk have shown lack of uniformity, owing to differences in technique and in standards of biological response used by different research workers.

Aykroyd and Roseoe (1), using (winter) cow's milk as the sole source of vitamin G, reported that an intake of 3 to 6 mls. (daily) produced gains of 9 and 12 grams per week (respectively) during a four week curative period, while Hunt and Krauss (2) observed that 5 mls. of milk were necessary to furnish sufficient vitamin G to produce a weekly gain in weight of 7 to 11 grams. Samuels and Koch (3) state that 25 mls. of milk were necessary to produce optimal growth when used as the sole source of vitamin B, while 17 to 20 mls. were required to furnish sufficient vitamin G. Todhunter (4) found that 9.3 mls. per week of pasteurized milk furnished sufficient vitamin G to produce a gain of 3 grams per week during an eight week experimental period. Krauss, Erb, and Washburn (5) state that about 25 per cent of the original vitamin B in milk is destroyed during ordinary pasteurization but that vitamin G is thermostable under these conditions. These writers found that 7.5 mls. of cow's milk were necessary to furnish one Sherman unit of vitamin B, while one unit of vitamin G was furnished by 5 mls. of the same milk. MacLeod, Brodie, and Macleod (6) reported that they found that the vitamin B and the vitamin G content of milk tended to remain fairly constant throughout the year and that the average content of vitamins B and G was 0.1 and 0.3 units (respectively) per gram of milk.

The studies reported in the present paper were initiated with the hope of obtaining further information regarding the vitamin B and vitamin G content of milk obtained from cows fed under carefully controlled conditions and to study the thermostability of these vitamins when subjected to various methods of pasteurization.

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EXPERIMENTAL

The preparation, composition, and handling of the basal diet used throughout this investigation were the same as that reported in a previous publication from this laboratory (7).

The vitamin supplement was prepared by percolating acidified commercial ethyl alcohol (5 mls. of concentrated HCl per liter of alcohol) through dried brewer's yeast as long as any color was removed from the yeast by this solvent. The percolate was concentrated under diminished pressure until it possessed the consistency of a thick syrup. The concentrated extract was cooled, placed in a separatory funnel and, after being diluted with one-half its volume of distilled water, was shaken with three separate portions of ethyl ether in order to remove the fatty materials. The fat-free extract was again concentrated under diminished pressure until 1 ml. of this concentrate represented the extract from approximately 20 grams of yeast. The excess acid was partially neutralized (pH of approximately 5.0) by carefully adding small quantities of a 10 per cent solution of sodium hydroxide. The concentrate was then diluted with alcohol to such a volume that 0.1 ml. of the resulting solution represented 1 gram of the original yeast. This extract was maintained at a sub-zero temperature for twenty-four hours after which it was rapidly filtered through a small Buchner funnel by means of suction, and preserved in a refrigerator until used.

The vitamin G supplement was prepared from dried baker's yeast by moistening the yeast with a saturated solution of sodium bicarbonate until a slightly alkaline product resulted. The alkalized yeast was autoclaved for six hours at 10 pounds pressure, after which it was dried and pulverized.

The milk used throughout the investigation was purchased from the college creamery. It was produced by the college herd and was of "certified" grade. The college herd received a uniform diet composed of a complex grain mixture, supplemented by alfalfa hay, ensilage and salt. The quantity of grain-mixture fed to each cow was proportional to the body weight of the animal and to the amount of milk produced, while each cow received eight pounds of alfalfa hay and twenty-two pounds of ensilage daily. The milk was produced under the most favorable sanitary conditions, and was cooled to a temperature of 36 to 40° F. immediately after milking. The raw milk was tested for its vitamin B and vitamin G potency as produced and again after each of four different heat treatments. The four different heat treatments employed were as follows: (I) ordinary pasteurization, (II) pasteurization under reduced pressure, (III) pasteurization with aeration, and (IV) by boiling for ten minutes.

In the first method (I) of pasteurization the milk was placed in an Erlenmeyer flask and stoppered with a one-hole rubber stopper, through which projected a thermometer. The flask of milk was placed in a water-

bath and the temperature of the milk was raised to 62–63° C. and maintained at this temperature for thirty minutes.

The second method (II) was very similar to the first except that a two-hole stopper containing a thermometer and a glass stopcock was used. When the temperature of the milk had reached 62–63° C., the stopcock was connected to a water pump and the pressure in the flask was reduced until the milk began to boil sufficiently to fill the flask with froth. The stopcock was then closed and the milk was maintained at this temperature for thirty minutes.

In the third method (III) a two-hole stopper containing a thermometer and a urea aeration tube was employed. After the milk had been heated to a temperature of 62–63° C., the aeration tube was connected to a water pump and the flow of water was so adjusted that a steady stream of air bubbles was drawn through the milk during the thirty minutes pasteurization period.

In the fourth method (IV) of pasteurization, the milk was placed in a round bottomed flask, which in turn was connected to a reflux condenser. The milk was heated to boiling on an electric hot plate and was maintained at this temperature for ten minutes.

Equal portions of fresh raw milk were pasteurized by the above methods twice weekly (Tuesday and Saturday) during the test period. Immediately after the pasteurizing treatment, each sample of milk was cooled rapidly by means of cold water, and was stored in an electric refrigerator until used. A corresponding sample of untreated milk was similarly preserved and was fed as a positive control.

Rats 20 to 21 days of age and weighing from 39 to 45 grams were placed in individual metal cages provided with raised screen bottoms, and were fed liberal quantities of the basal diet. The animals were weighed at weekly intervals, at which time records were made of food consumption and physical condition. After the animals began to lose weight (usually from 12 to 20 days after being placed on the diet), they were divided into groups, care being taken to compensate so far as possible for both sex and litter variations. At this time the basal diet of each animal, excepting that of those retained as negative controls, was supplemented daily by definite quantities of milk, of the vitamin B concentrate or of the vitamin G fraction, either alone or in some definite combination. Each supplement to the basal diet was fed daily in separate containers for a period of at least six weeks, unless the test animal died previous to the completion of the experiment.

The first part of the investigation was restricted to testing the vitamin B concentrate, the vitamin G fraction, and the raw milk for their vitamin B complex potency. To do this, a total of 36 depleted animals was used. Six of these animals were retained on the unsupplemented basal diet to serve

as negative controls. Another group of six animals received the basal diet and, in addition, a daily supplement of 0.1 ml. of the vitamin B concentrate. A third group of six animals received (daily) 0.3 grams of autoclaved yeast as a supplement to the basal diet. A fourth group of six animals received both the vitamin B concentrate and the vitamin G fraction as supplements to the basal diet. The twelve remaining animals were divided into six groups of two animals each, and the rats in the respective groups received, as a supplement to the basal diet, 1, 2, 3, 4, 6, and 8 mls. of raw milk (V) daily. The responses made by these groups of animals are given in Table 1 and Chart 1.

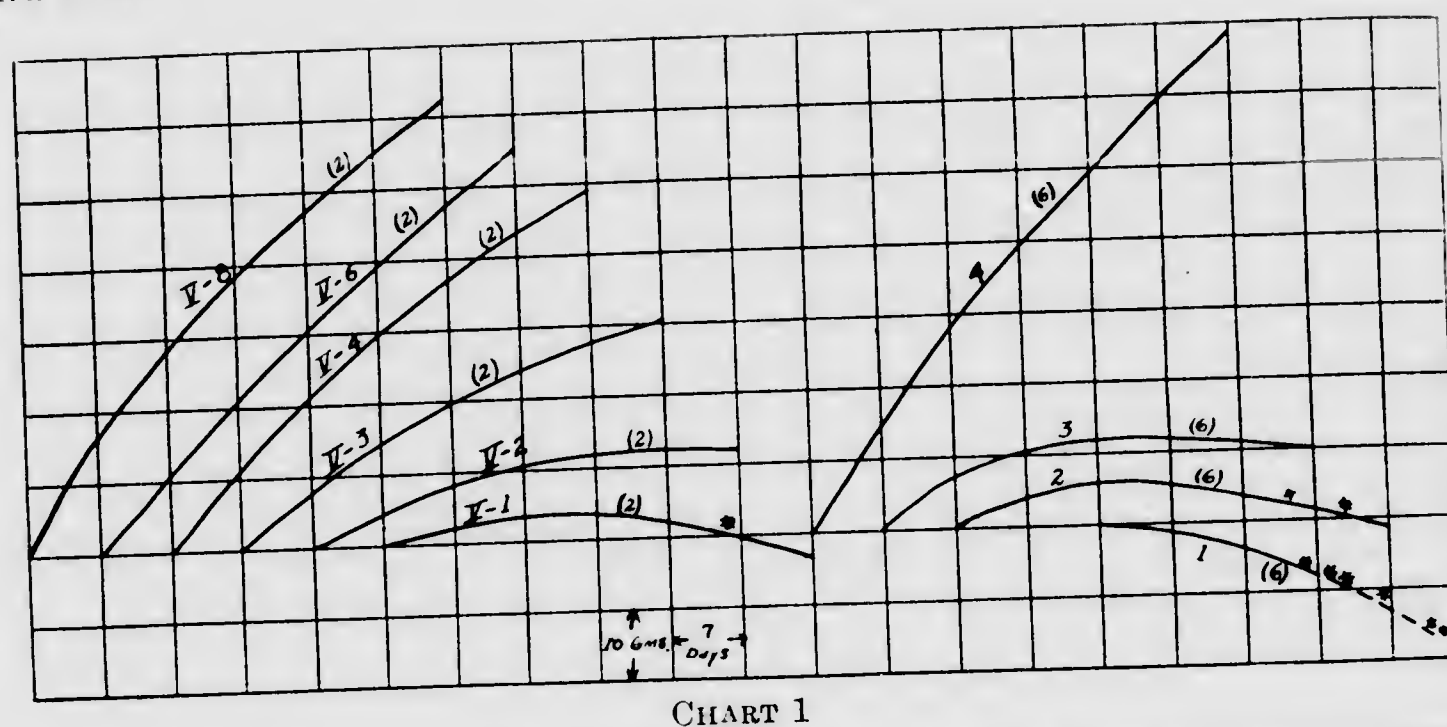


CHART 1

Showing the average growth responses made by the various groups of animals receiving the basal diet unsupplemented (Curve 1); supplemented with vitamin B concentrate (Curve 2); supplemented by the vitamin G fraction (Curve 3); supplemented by both vitamins B and G (Curve 4); and supplemented by definite quantities of unpasteurized milk (Curves V-1 to V-8). The "V" curves represent growth responses made by animals receiving milk as the only source of vitamins B and G. The number immediately following the "V" indicates the quantity of milk fed. The number in parenthesis indicates the number of animals considered. The asterisk indicates the death of an animal.

The data obtained showed that 3 mls. of the unpasteurized milk were sufficient to produce a gain of 30 grams during a six-week experimental period, when used as the sole source of the vitamin B complex. In consequence of this fact, a 3 mls. daily dosage was chosen as the amount to be fed in the study relative to the vitamin B and the vitamin G content of this milk before and after the several methods of pasteurization. Groups of animals (varying from 3 to 12 animals per group) were fed 3 mls. daily of one of the five types of milk to be tested, alone, and in combination with the vitamin B concentrate and the vitamin G fraction. An outline of this phase of the investigation, together with the resultant growth are given in Tables 1 and 2, and in Chart 2.

TABLE 1
Showing the quantities of the vitamin supplements used, the number of animals considered, their average initial weight, average weight at end of depletion period, average weekly gain, and average weekly food consumed

DIET NO.	MILK SUPPLEMENT	QUANTITY OF MILK FED	VITAMIN B CONCENTRATE FED	VITAMIN G FRACTION FED	NO. OF RATS CONSIDERED	AVERAGE INITIAL WEIGHT	AVERAGE WEIGHT AT END OF DEPLETION PERIOD	GAIN						AVERAGE WEEKLY FOOD CONSUMPTION
								1st week	2nd week	3rd week	4th week	5th week	6th week	Total
306					6	42	49	-3	-1	-3	-6	-1	-2	0
"			0.1		6	41	48	7	4	2	0	-3	-4	10
"				0.3	6	41	49	4	3	0	-2	10	8	67
"			0.1	0.3	6	42	48	15	13	11	10	10	8	67
"	V	1			2	41	49	3	1	0	-1	-2	-1	13
"	V	2			2	41	48	5	3	5	5	6	5	30
"	V	3			2	41	51	7	7	9	7	8	7	36
"	V	4			2	42	48	11	10	10	9	8	8	36
"	V	6			2	43	50	16	12	10	9	6	6	36
"	V	8			2	43	50	8	5	6	4	3	3	36
"	V	3	0.1		6	43	50	8	10	9	7	6	4	35
"	V	3		0.3	6	43	51	5	3	3	3	3	3	20
"	V	3	0.1	0.3	11	43	51	8	7	5	5	4	3	24
"	V	3		0.3	3	43	51	6	3	3	3	3	3	19
"	V	3	0.1	0.3	11	41	48	6	6	4	4	3	3	24
"	V	3		0.3	3	42	49	6	6	4	4	3	3	21
"	V	3	0.1	0.3	10	43	50	4	7	6	6	3	3	30
"	V	3		0.3	3	43	51	8	7	6	6	3	3	24
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
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"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21
"	V	3	0.1	0.3	11	43	51	7	7	6	6	3	3	24
"	V	3		0.3	3	43	51	6	6	4	4	3	3	21

TABLE 2
Showing the average gains in weight in grams made by the several groups of animals receiving 3 mls. daily of the various milks, and milks supplemented by the Vitamin B concentrate and the Vitamin G fraction, respectively

	UNPASTEURIZED MILK (V)	PASTEURIZED BY USUAL METHOD (I)	PASTEURIZED UNDER PARTIAL VACUUM (II)	PASTEURIZED WHILE AERATED (III)	BOLLED FOR 10 MINUTES (IV)
Milk alone	26 gms. (100%)	20 gms. (77%)	16 gms. (62%)	21 gms. (81%)	22 gms. (85%)
Milk with vitamin B	43 gms. (100%)	37 gms. (86%)	27 gms. (63%)	34 gms. (79%)	37 gms. (86%)
Milk with vitamin G	35 gms. (100%)	27 gms. (77%)	26 gms. (74%)	30 gms. (86%)	34 gms. (97%)

For convenience, the data are also expressed in terms (percentage) of the responses made by groups of animals receiving the untreated milk, alone and similarly supplemented. It is realized that values obtained by this method of calculation are only approximations. More accurate results could have been obtained if definite quantities of each milk could have been fed to produce a unit gain in weight.

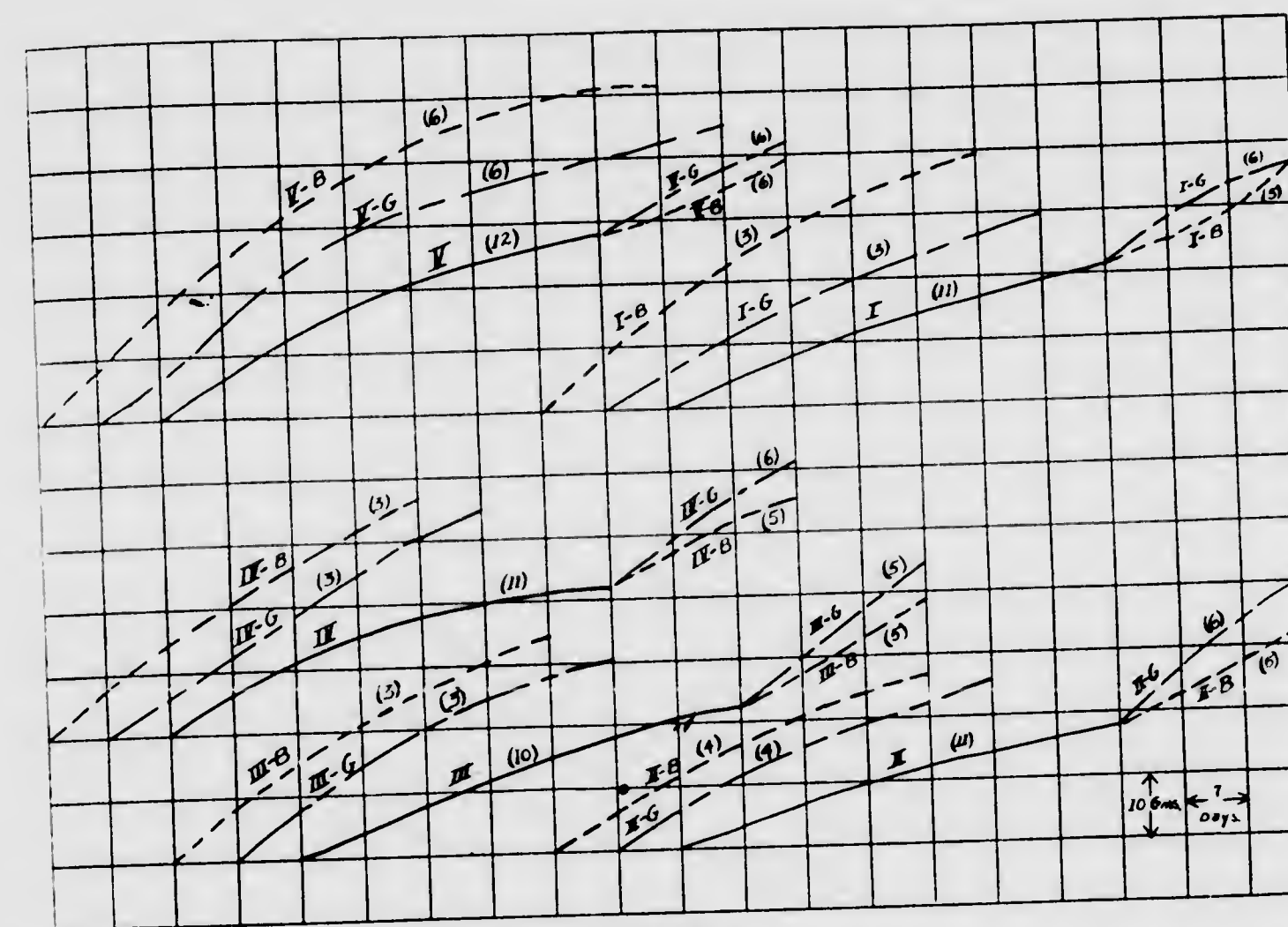


CHART 2

Showing the average growth responses made by groups of animals receiving 3 mls. daily of the various milks, alone and in combination with the vitamin B concentrate and the vitamin G fraction, respectively. The Roman numeral indicates the method of pasteurization, the letter indicates the additional supplement added, and the number in parentheses indicates the number of animals considered.

- I = ordinary pasteurization
- II = pasteurization under partial vacuum
- III = aerated while pasteurized
- IV = pasteurized by boiling for 10 minutes
- V = unpasteurized
- B = vitamin B concentrate
- G = vitamin G fraction

Those groups which received one of the five samples of milk unsupplemented by either the vitamin B concentrate or the vitamin G fraction, and which survived a seven-week experimental period, were divided into two groups of approximately equal number. The animals of one group were given, in addition to the usual milk supplement, 0.1 ml. of the vitamin B concentrate daily, while the animals of the other group received 0.3 grams of the vitamin G fraction daily. These extra supplements were fed for three additional weeks. The results obtained by adding further supplements at this stage of the investigation are shown by the extended growth curves (broken lines) in Chart 2.

DISCUSSION

While the experimental data here presented are compiled from the records of 120 animals, a considerably larger number of animals was used

in the investigation. These data are representative of those obtained as a result of three series of tests carried on at different seasons of the year. The first series of tests was begun in June, while the second and third series were started in October and March, respectively. Since the responses made by corresponding groups of animals during the three seasons were so nearly identical, the data have been presented in a composite form, using the records of only those animals which reacted most typically in their respective groups. Records of experimental animals which were known to have depleted their vitamin stores and of those animals which were improperly depleted and resorted to any degree of coprophagy during the test period were eliminated. Efforts were made to use experimental animals of approximately the same initial weight in the various groups and during the three series of tests. As has been previously stated, these animals were distributed among the several groups so as to compensate for both litter and sex variations. A majority of the animals which were placed on the basal diet, for the depletion period, gained from 5 to 8 grams during the first 9 to 14 days, after which their weight became stationary, and in many cases a slight loss in weight ensued. It was found to be undesirable to continue the depletion period for more than 20 to 21 days because of the high incidence of spontaneous re-growths due to coprophagy, which is frequently practiced and observed among over-depleted rats receiving a basal diet of this type. Growth responses made by such animals are unreliable as indices of the vitamin B or the vitamin G potency of a food material.

The control animals which received the basal diet unsupplemented (Curve 1, Chart 1) continued to decline in weight and a majority of them were dead before the end of the fourth week of the experimental period. Some manifestations of beriberi were usually observed in each case, but the paralytic symptoms were more pronounced among those animals which survived the longest, unless such animals were practicing some degree of coprophagy.

The depleted animals which received the basal diet supplemented by the vitamin G fraction (Curve 2, Chart 1) made a slight response in growth during the first two weeks in which the supplement was fed, and then showed a gradual loss in weight until the end of the experiment. Most of these animals showed marked paralytic symptoms, especially during the sixth week, and a number of them died during this time.

Animals receiving the basal diet, supplemented by daily additions of the vitamin B concentrate (Chart 1, Curve 3), made a slow but definite growth response for the first three weeks, which was followed by a slight loss in weight during the following three weeks. By this time most of the animals were beginning to manifest some of the symptoms usually ascribed to vitamin G deficiency.

Those animals which received the basal diet supplemented by both the vitamin B concentrate and the vitamin G fraction (Curve 4, Chart 1) made an average gain in weight of 11 grams per week during the six-week period. These animals appeared normal in all respects with the exception of the abnormally dry condition of their skin and hair.

Curves V-1 to V-8 (Chart 1) show the growth response made by animals receiving varying quantities of the unpasteurized milk as a supplement to the basal diet. The quantities of milk fed daily were 1, 2, 3, 4, 6, and 8 mls., respectively. Although 1 or 2 mls. of milk daily had a definite supplemental effect on the basal diet, the animals which received these quantities of milk frequently manifested symptoms of beriberi. When the milk supplement was increased to 3 mls. daily, a fairly uniform gain in weight resulted throughout the six-week period, and none of the animals showed characteristic symptoms of vitamin B deficiency. While still greater daily allotments of milk resulted in more marked growth responses and further improvements in the general appearance of the animals, it was believed that a growth response similar to that produced by 3 mls. of milk daily would be the most accurate in evaluating the vitamin B and the vitamin G content of milk which had been pasteurized by any one of the several methods described.

Curve V (Chart 2) shows the growth response made by a larger group of animals which received (daily) 3 mls. of unpasteurized milk as a supplement to the basal diet. These animals made an average gain of 4 grams per week, and appeared to be in a fairly thrifty condition throughout the experiment. When the basal diet was further supplemented by either the vitamin B concentrate or the vitamin G fraction, in addition to the 3 mls. of unpasteurized milk, further growth resulted in each case (Curves V-B and V-G), but the vitamin B concentrate excelled the vitamin G fraction as a growth producing supplement under these conditions.

Curve I (Chart 2) shows the growth response made by a group of animals receiving the basal diet supplemented daily by 3 mls. of milk pasteurized by ordinary pasteurization (Method I). These animals showed an average gain of approximately 3 grams per week during the 6-week period. Additional growth resulted when the diet was further supplemented by the vitamin B concentrate and by the vitamin G fraction (Curves I-B and I-G), but here again the vitamin B concentrate excelled in supplementing value.

Curve II (Chart 2) shows the average growth response made by a group of 11 animals that received (daily) 3 mls. of milk which had been pasteurized under a partial vacuum (Method II). The average growth rate in this case was less than 2.7 grams per week, which is considerably less than the gains made by those animals which received the unpasteurized milk under similar conditions (Curve V). When calculated on the basis of

growth responses produced before and after pasteurization, only 62 per cent of the original vitamin B complex was retained by the milk after being pasteurized in this manner. In this case the vitamin B concentrate proved to have a marked supplemental value when fed in addition to the milk supplement (Curve II-B), while additions of the vitamin G fraction resulted in equally definite increments of growth (Curve II-G).

We are unable to offer any explanation as to why milk pasteurized by this method contains less of these vitamins than similar milk pasteurized in the usual manner (Method I). In fact, these findings were contrary to our expectations. Since similar differences were observed in three series of experiments carried on during different seasons of the year, it appears that the results are beyond the realm of chance. Some attempts were made, therefore, to arrive at a plausible explanation for the apparent destruction or inactivation of these vitamins, but no definite conclusion could be drawn from the data obtained. It does not seem probable that such destruction could be the result of any slight change in hydrogen-ion concentration of the milk as the result of the partial removal of the dissolved gases while under partial vacuum; yet this is a phase of the problem that we hope to study as soon as time will permit.

The group of animals which received the milk that had been aerated during the process of pasteurization (Method III) made an average gain of 3.5 gms. per week (Curve III, Chart 2). While these results indicate that some destruction of the vitamin B complex had taken place, this milk compared favorably with that produced by the usual method of pasteurization (Curve I). When this diet was further supplemented by either vitamin B or vitamin G fractions, additional responses in growth were obtained (Curves III-B and III-G). Of the two factors, the vitamin B concentrate proved slightly superior as a growth stimulator under these conditions.

When raw milk, which had been boiled for 10 minutes under a reflux condenser, was fed to a group of rats as a supplement to the basal diet, it was found to have retained the greater portion of its original vitamin B complex content (Curve IV, Chart 2). In fact, this milk appears slightly superior in this respect to milks pasteurized by either Methods I, II or III. Those groups of animals which received further dietary supplements in the form of either vitamin B concentrate or vitamin G fraction grew at an increased rate (Curves IV-B and IV-G). In this dietary combination the vitamin B concentrate proved more effective in stimulating additional growth than did the vitamin G fraction.

When each of the five groups of animals which had been receiving the five different milks as the sole supplement to the diet (Groups I, II, III, IV and V) were divided at the end of the seventh experimental week, one half of each group being given daily allotments of the vitamin B concen-

trate and the other half of each group being given the vitamin G fraction in addition to the usual daily allotment of the respective milks, some interesting growth responses were observed (see broken line extensions of Curves I, II, III, IV and V). At this stage of the experiment, the vitamin G supplement proved to be the most effective growth-stimulator in every case. These results are not in agreement with those which had been obtained by means of similar groups of animals of less mature age, during the preceding weeks. But the consistency of growth-trends in this connection is sufficient at least to suggest that the relative vitamin B and vitamin G requirements of the rat vary with variations in stage of maturity.

SUMMARY

1. Experiments are described in which raw certified milk, produced by the college dairy herd, was fed to rats with and without supplementation with potent preparations of vitamins B and G.
2. At three different periods during the year these experiments were repeated, at which times the raw milk was pasteurized by four methods, *viz.*, (1) ordinary pasteurization at 62-63° C. for 30 minutes; (2) same as (1) except that pasteurization was conducted under reduced pressure; (3) same as (1) except that the milk was aerated; and (4) boiled for 10 minutes under a reflux condenser.
3. The pasteurized milks were also fed to rats in the presence and in the absence of potent preparations of vitamins B and G and comparisons were made with raw milk from which the pasteurized milks were made.
4. It was found that the raw milk contained appreciable quantities of vitamins B and G and that 3 mls. per day were sufficient to furnish at least one Sherman unit of each of the vitamins.
5. It was found that vitamin B was the limiting factor when 3 mls. of raw milk were fed as the sole source of vitamins B and G but the data indicate that the B potency was not limited to the extent that has been reported by certain investigators.
6. It was observed that the vitamin B and G potency of the raw milk was remarkably constant throughout the year.
7. Three separate sets of experiments showed greater loss of vitamin B and vitamin G when the milk was pasteurized under diminished pressure. This was contrary to expectations and will be investigated in the near future.
8. Some loss of vitamins B and G occurred in all methods of pasteurization but less destruction occurred when milk was boiled for 10 minutes under a reflux condenser.
9. Although the maximum destruction of either vitamin B or vitamin G, as a result of any one of the four methods of pasteurization, was about 38 per cent (calculated from differences in growth response), the destruction

of these vitamins under carefully controlled plant operation need not be as great as indicated above.

10. It is suggested that the relative requirements for vitamin B and vitamin G by the rat vary with the age of the rat, the evidence suggesting that the requirement for vitamin B is greater in the young rat and that the need for vitamin G seems to be greater as the rat matures.

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Changes in Caudal Bones of the Rat as an Index of Ossification.*

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The conventional method of determining the rachitic condition of a rat by either the X-ray or the "line test" method has been restricted primarily to an examination of the longer bones of the rat's body, especially the proximal end of the tibia or distal end of the femur. While a study of the structural changes in such bones furnishes a reliable index of the degree of rickets manifested by the animal in question, a comparison of X-ray photographs of the whole body of such animals indicated that other bones of the skeletal framework might offer some advantages over the tibia and the femur in this respect. This appeared to be especially true of the bones of the tail. This particular body structure appeared to offer a series of provisional zones of calcification which could be studied by either the X-ray or the "line test" method. In fact the tail of the rat had been found to be both more easily and more effectively X-rayed than was the leg and, in addition, it afforded possibilities of removing portions of the bony segments for "line test" during the course of the experimental period, without serious consequence to the health and well-being of the experimental animal.

In connection with another investigation being carried out in this laboratory in which a large number of rachitic animals were being involved, a comparison was made of the X-ray and the "line test" findings, where both the tibia and the caudal bones were considered. A general idea of the comparative results obtained may be had by observing Plates 1 and 2, which were obtained from X-rays and

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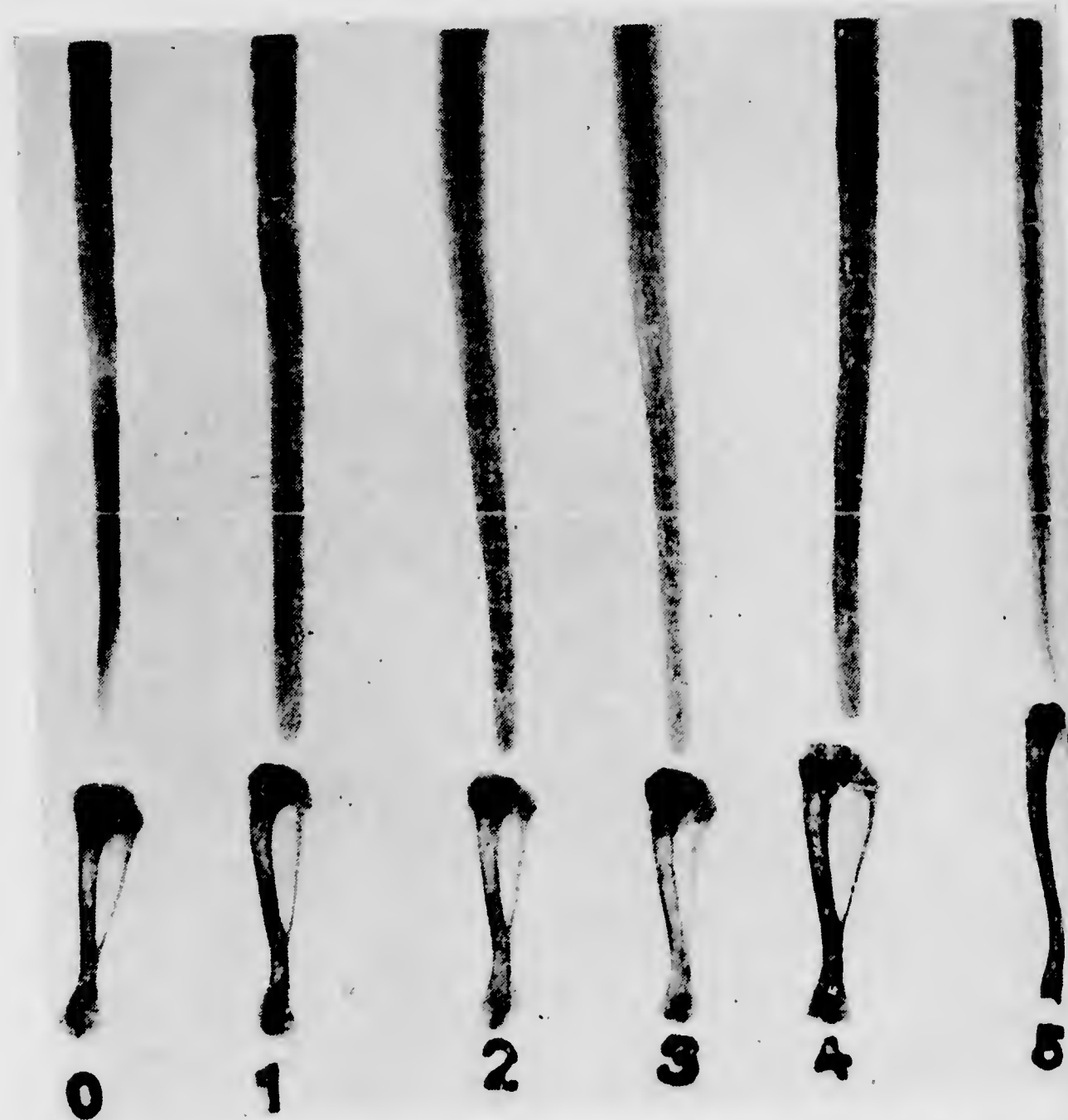


PLATE 1, showing X-ray photographs of both the tibiae and the tails of typical animals, each representing a different stage of ossification.

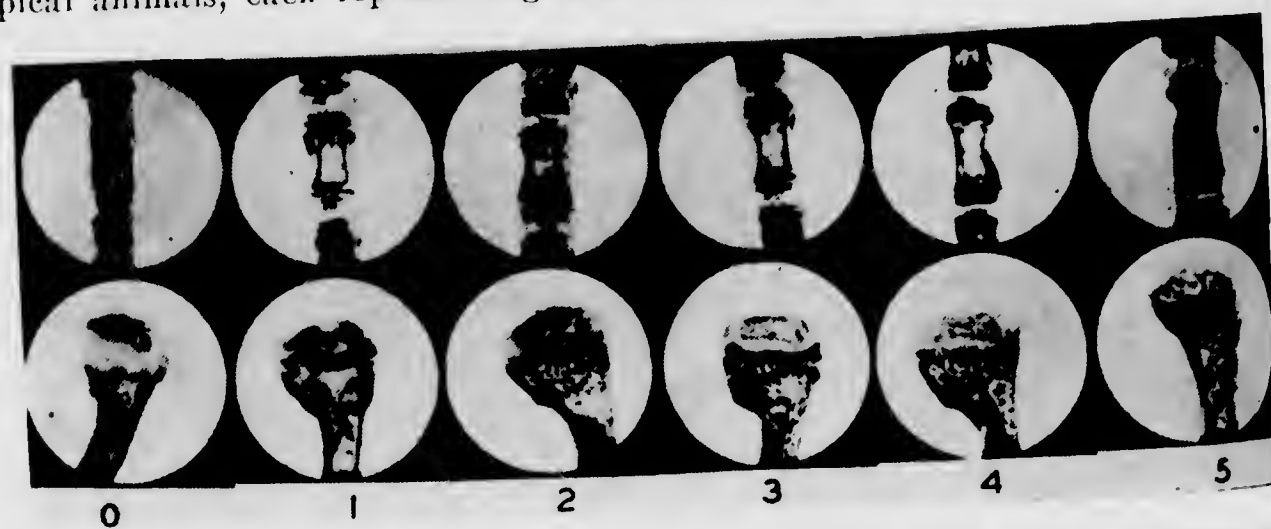


PLATE 2, showing photographs of "line tests" on a tibia and a section of caudal bones taken from each of the 6 animals considered in Plate 1.

"line test" photographs of the bones of 6 typical animals that represented various stages of ossification.

Animals 0 to 4 inclusive were placed on a rickets-producing diet when 21 days of age. At this time their weights ranged between

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and 46 gm. After being on the rachitogenic diet for 21 days, animal 0 was killed as a negative control, while the 4 remaining animals received one unit (A.D.M.A.) of vitamin D daily as a supplement to the rachitogenic diet. Animal 1 was killed on the 31st day, animal 2 on the 35th day, animal 3 on the 38th day and animal 4 on the 42nd day. Animal 5 was taken directly from the breeding colony and was 54 days of age when killed.

The tibia and the tail were removed from the dead animal and preserved in a 50% alcohol-water solution until the desired photographs could be made. For the X-ray photographs, the 6 tibiae and the 6 tails were arranged above a single X-ray photographic plate and X-rayed simultaneously. For the "line test" photographs, the bones were prepared in the usual manner and individual photographs were taken.

Through such photographs and other observations, we are led to believe that both X-ray and "line test" made of the caudal bones of the rat are reliable indices of the degree of ossification. The caudal bones appear to offer some advantages over the tibia or the femur in certain respects, but do require greater pains in the preparation for "line testing" than does either the tibia or the femur. Some of these advantages are: (a) the caudal bones are both more easily and more effectively X-rayed, (b) the caudal bones offer a series of zones of calcification instead of a single zone, and (c) the caudal bones offer possibilities of removing segments for "line test" during the progress of the experiment. While such advantages may be important considerations in certain phases of research, it is not suggested that the caudal bones replace the tibia or the femur in the conventional method of determining the state of ossification.

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VITAMIN STUDIES

XIX. THE ASSIMILATION OF CAROTENE AND VITAMIN A IN THE PRESENCE OF MINERAL OIL

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SEVEN CHARTS

(Received for publication January 20, 1934)

In a previous paper from this laboratory (Dutcher, Ely and Honeywell, '27), experiments were described which indicated that rats are unable to utilize vitamin A in butter fat when the latter is dissolved in mineral oil. At that time we concluded that "mineral oil may act as a solvent for vitamin A thereby depleting the ingested foods of their supply of this vitamin." Our findings were in general agreement with those of Burrows and Farr ('27) who postulated that unabsorbed mineral oil might act as a solvent for vitamin A in the food and in the mucosa of the digestive tract.

Subsequent experiments in our laboratory indicated quite clearly that the above generalization was not applicable to vitamin A in cod liver oil, since the latter (in mineral oil) seemed to be utilized more efficiently than butter fat when fed at corresponding levels and under similar conditions. These results substantiated the observations of Moness and Christianson ('29), who described experiments in which a concentrate prepared from cod liver oil was dissolved in olive oil and in mineral oil, respectively. These authors concluded

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that the utilization of vitamin A from cod liver oil is not appreciably affected by the presence of mineral oil.

Rowntree ('31), working with cod liver oil, concluded that the utilization of vitamin A is lowered by mineral oil only when the cod liver oil is fed at relatively low levels. She expressed the belief that mineral oil can be used with impunity if the vitamin A content of the diet 'is adequate.' Since our interest in the problem relates solely to the question of the use of mineral oil as a vehicle or solvent for vitamin A in the biological assay of food materials, we have purposely confined our vitamin A intakes to those levels which would be considered relatively low.

Jackson ('31) repeated our experiments, using minimal doses of butter fat, and concluded that mineral oil tends to prevent the utilization of vitamin A when butter fat and mineral oil are mixed prior to ingestion, but that practically no detrimental effects can be noted when butter fat and mineral oil are administered separately.

The relatively recent discovery (Moore, '29, '30) that the pigment carotene is the precursor or parent substance of vitamin A seemed to offer a possible explanation for the apparently diverse results obtained with pigmented butter fat and non-pigmented cod liver oil.

The experiments described in the present paper were undertaken to determine the effect of mineral oil on the utilization of, a) purified carotene, b) a pigment-free cod liver oil, and, c) a vitamin A concentrate prepared therefrom, and to ascertain, if possible, the mechanism whereby mineral oil lowers the vitamin potency of butter fat.

EXPERIMENTAL

The rats used in this study were placed on experiment at 21 days of age, at which time they averaged about 40 gm. in weight. All animals were maintained in individual metal cages throughout the entire experimental period. The cages were equipped with false bottoms consisting of heavy wire screening containing two meshes to the inch.

The vitamin A-free ration consisted (in parts per 100) of casein 18, agar 2, dextrin 77, and McCollum and Simmond's no. 185 ('17) salt mixture 3. Vitamins B and G were furnished by daily allotments (0.6 gm.) of yeast which were fed separately from the basal ration to insure a constant and ample supply (Honeywell, Dutcher and Ely, '31) of these vitamins. The basal ration was irradiated with a carbon arc lamp at a distance of 18 inches for 20 minutes (Dutcher and Kruger, '26) to insure sufficient vitamin D for the experimental period.

All animals were depleted of their vitamin A reserves by feeding the basal diet until they showed evidence of vitamin A deficiency, i.e., by the appearance of incipient xerophthalmia or by loss of body weight. At this point the materials to be assayed were fed in measured amounts separate from the basal ration and the curative period was continued for 5 weeks. Evidence for the assimilation of sufficient amounts of carotene or vitamin was considered satisfactory when xerophthalmic symptoms disappeared and when the rate of growth averaged not less than 3 gm. per week during the 5-week curative period. Each experimental group consisted of at least five rats with the exception of a few groups which contained but three or four individuals per group, owing to shortage of animals.

The vitamin A concentrate used in this study was prepared by slight modifications of the method of Drummond, Channon and Coward ('25) which consisted essentially of saponification of 900 gm. of cod liver oil with alcoholic potassium hydroxide, extraction of the unsaponifiable fraction with ether, and evaporation of the ether in an atmosphere of carbon dioxide. Cholesterol was removed from the yellow semi-solid mass by freezing out in methyl alcohol, after which the alcoholic solution of vitamin A was evaporated in an atmosphere of carbon dioxide to a light yellow semi-solid fraction. This fraction, which weighed 0.5 gm., was dissolved in corn oil and made up to a volume of 500 ml. One cubic milliliter of this solution was again dissolved in corn oil and made up to a volume of

250 ml. One-half ml. of this solution was found to contain one Sherman unit of vitamin A, which is equivalent to 500,000 Sherman units per gram of concentrate.

The crystalline carotene used in this study was also dissolved in corn oil and all levels of carotene and vitamin A concentrate were adjusted so that all dosages were administered in $\frac{1}{2}$ ml. of corn oil (daily) in order that variations might be eliminated due to fluctuations in daily intakes of corn oil.

FIRST SERIES OF EXPERIMENTS

In the first series we repeated our former experiments with butter fat, which was fed at two levels, viz., 20 and 40 mg.

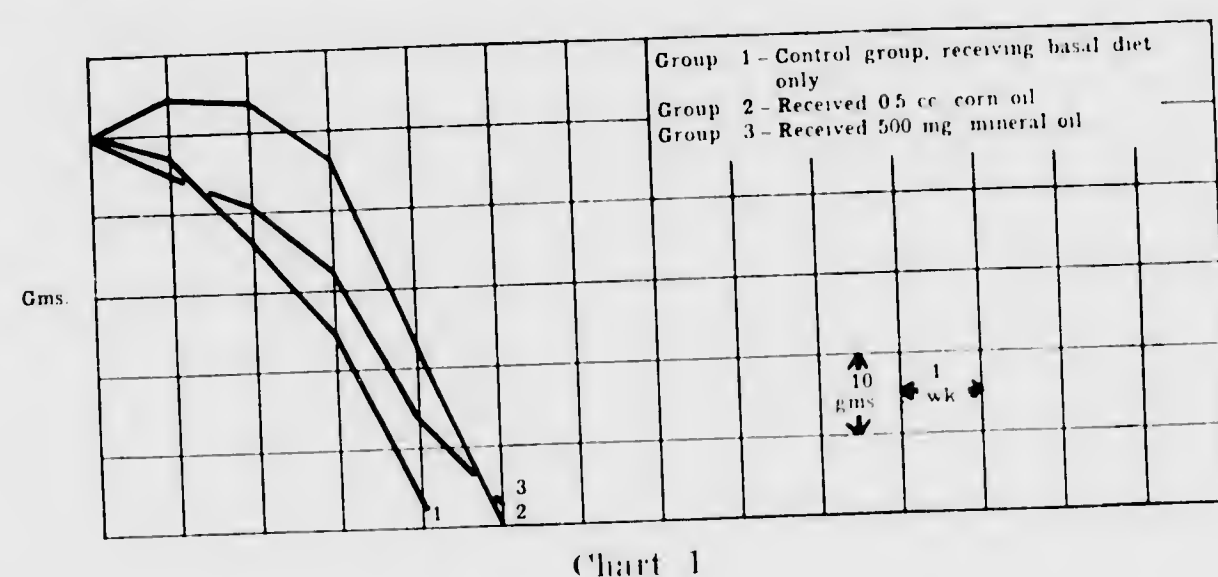


Chart 1

daily. In order to prove that the presence of corn oil had no detrimental effect, two control groups were fed vitamin A-free diets. One of these (group 2) received 0.5 ml. of corn oil (daily) during the curative period. A third control group received the basal diet with additions of 500 mg. of mineral oil (daily) throughout the curative period. The results obtained are summarized in chart 1.

In order to eliminate the possibility of loss of carotene or vitamin A by possible oxidative changes due to the presence of oxidants, two of the butter fat diets (groups 6 and 9) were protected by the anti-oxidant hydroquinone. The results of these experiments are summarized in chart 2.

Groups 10 to 20 inclusive were fed the basal ration supplemented with carotene during the curative period to establish the potency of the carotene employed. It will be noted (chart 3) that 2.5 γ of carotene (daily) were equivalent to one Sherman unit.

Chart 4 summarizes the data obtained when various levels of carotene were fed during the curative period in the presence

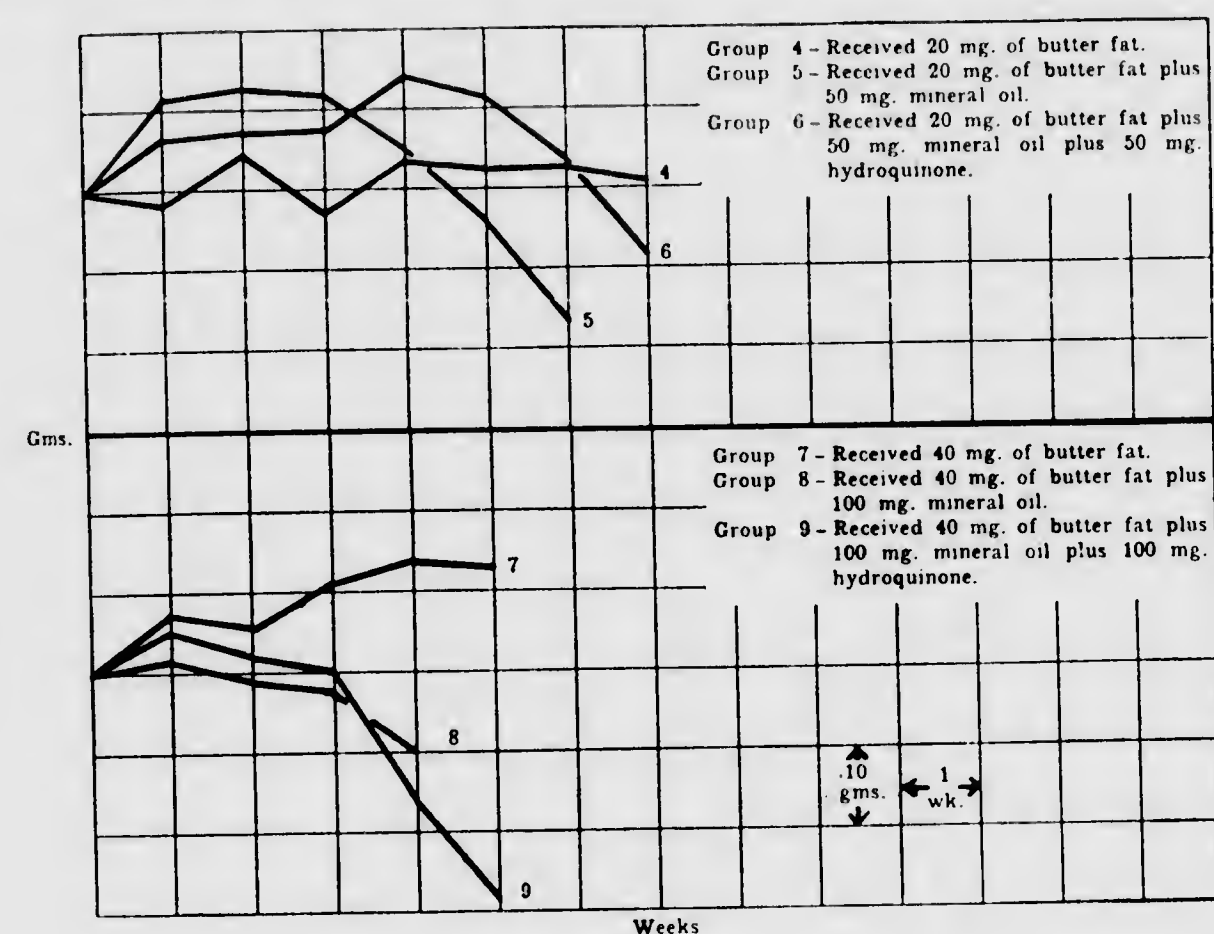


Chart 2

and absence of varying amounts of mineral oil and in the presence and absence of hydroquinone.

Groups 23 to 28 inclusive received varying daily allotments of cod liver oil in the presence and absence of varying amounts of mineral oil. These results are summarized in chart 5.

Chart 6 shows the results obtained when 2×10^{-6} mg. of cod liver oil concentrate were fed in the presence and absence of mineral oil.

Discussion of results obtained in first series of experiments

The results obtained with butter fat dissolved in mineral oil are in general agreement with those previously described

(Dutcher, et al., '27; Jackson, '31). It is clear that mineral oil, in some manner, prevents the utilization of carotene or vitamin A or both. The fact that hydroquinone (chart 2) did not prevent this deleterious effect would seem to support the postulation that the loss of vitamin A activity is not due to the presence of a pro-oxidant in mineral oil.

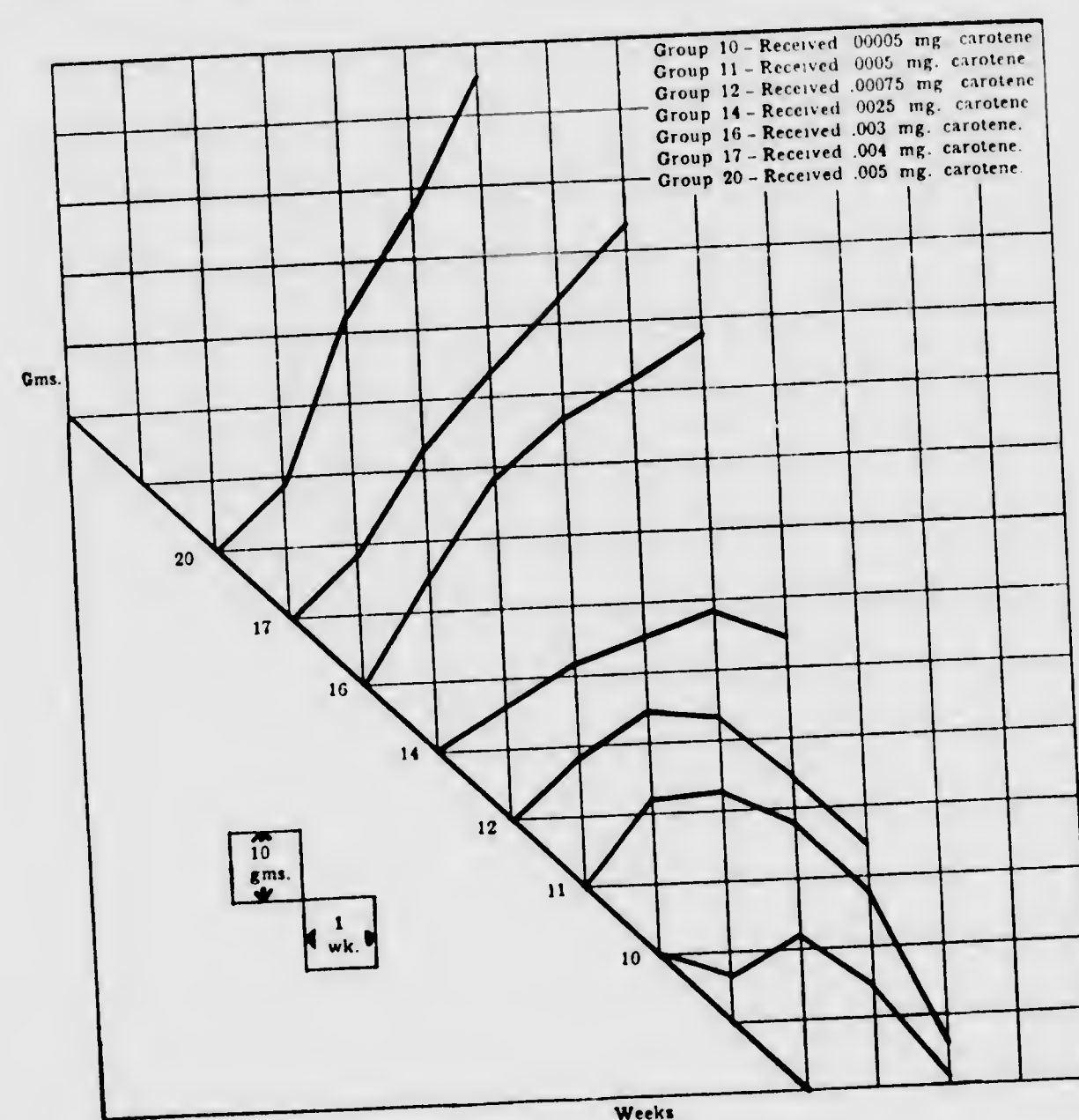


Chart 3

When carotene was fed as the sole source of vitamin A activity, the results were even more convincing (chart 4). When carotene was fed in daily doses of 2.5 γ , as little as 6.25 γ of mineral oil caused marked retardation of growth (groups 14 and 15). When carotene was fed in excess of the Sherman unit requirement (groups 17, 18 and 19), better

growth responses were obtained, but the effect of the small amount of mineral oil was still quite marked. It will be noted, also, that the presence of 100 mg. of hydroquinone did not prevent the deleterious effect of the mineral oil. When the dosage of carotene was increased to 5 γ , growth response was excellent, except where mineral oil was added. When an excessive amount of mineral oil (2 ml.) was fed daily in

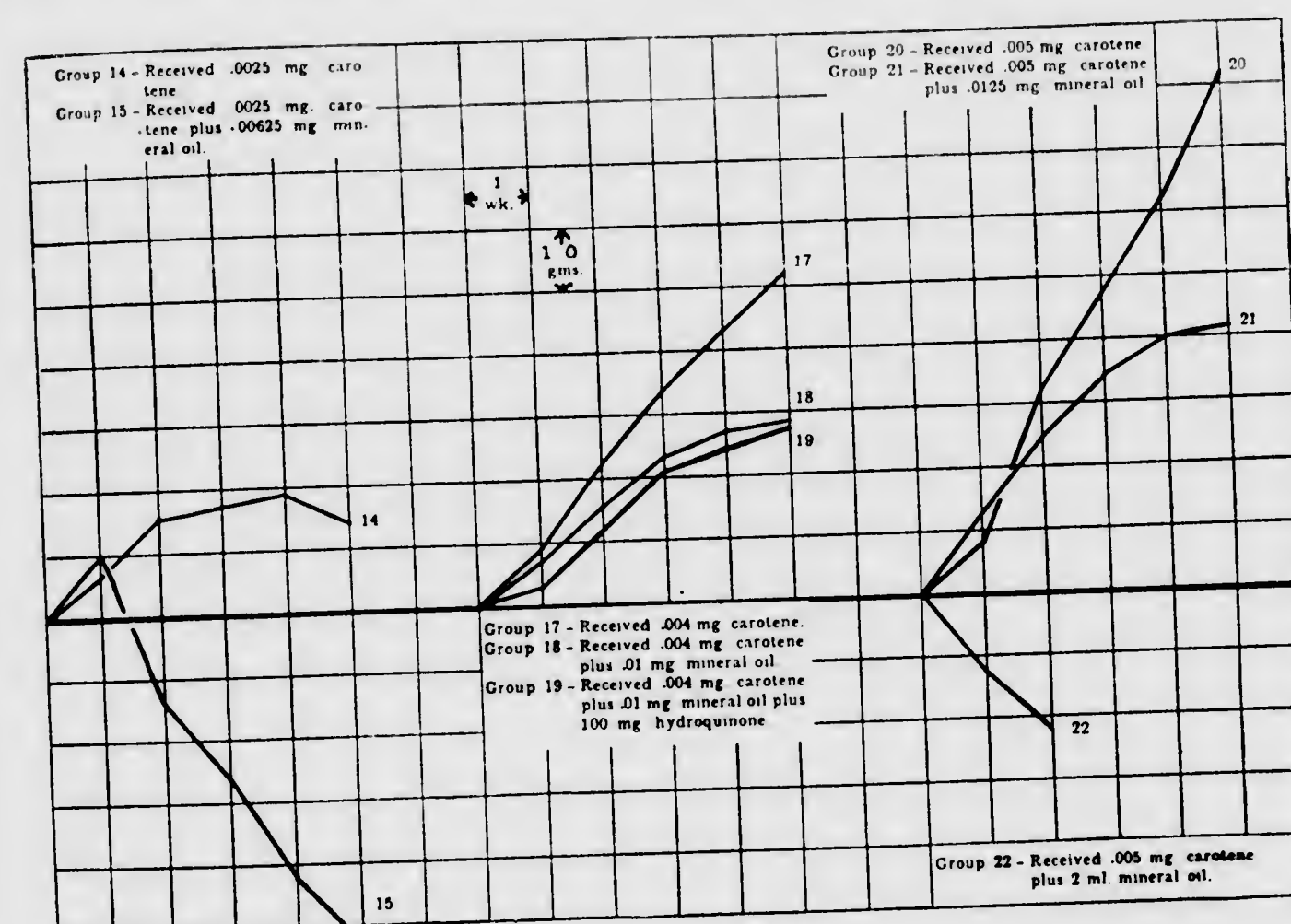


Chart 4

the presence of 5 γ of carotene (group 22), all animals died within a period of 2 weeks with the usual symptoms of vitamin A deficiency.

When cod liver oil was fed daily (chart 5) at a level of 0.6 mg., which was barely sufficient for maintenance of body weight, there was some evidence that vitamin A utilization was lessened by the presence of 500 mg. of mineral oil. When the dosage of cod liver oil was increased to 1 mg. (daily), which was sufficient for growth, the presence of 500 mg. of mineral oil showed no visible effect on growth response

(groups 25 and 26). Similar results were obtained with groups 27 and 28 when the daily allotments of cod liver oil and mineral oil were increased to 2 mg. and 2 ml., respectively.

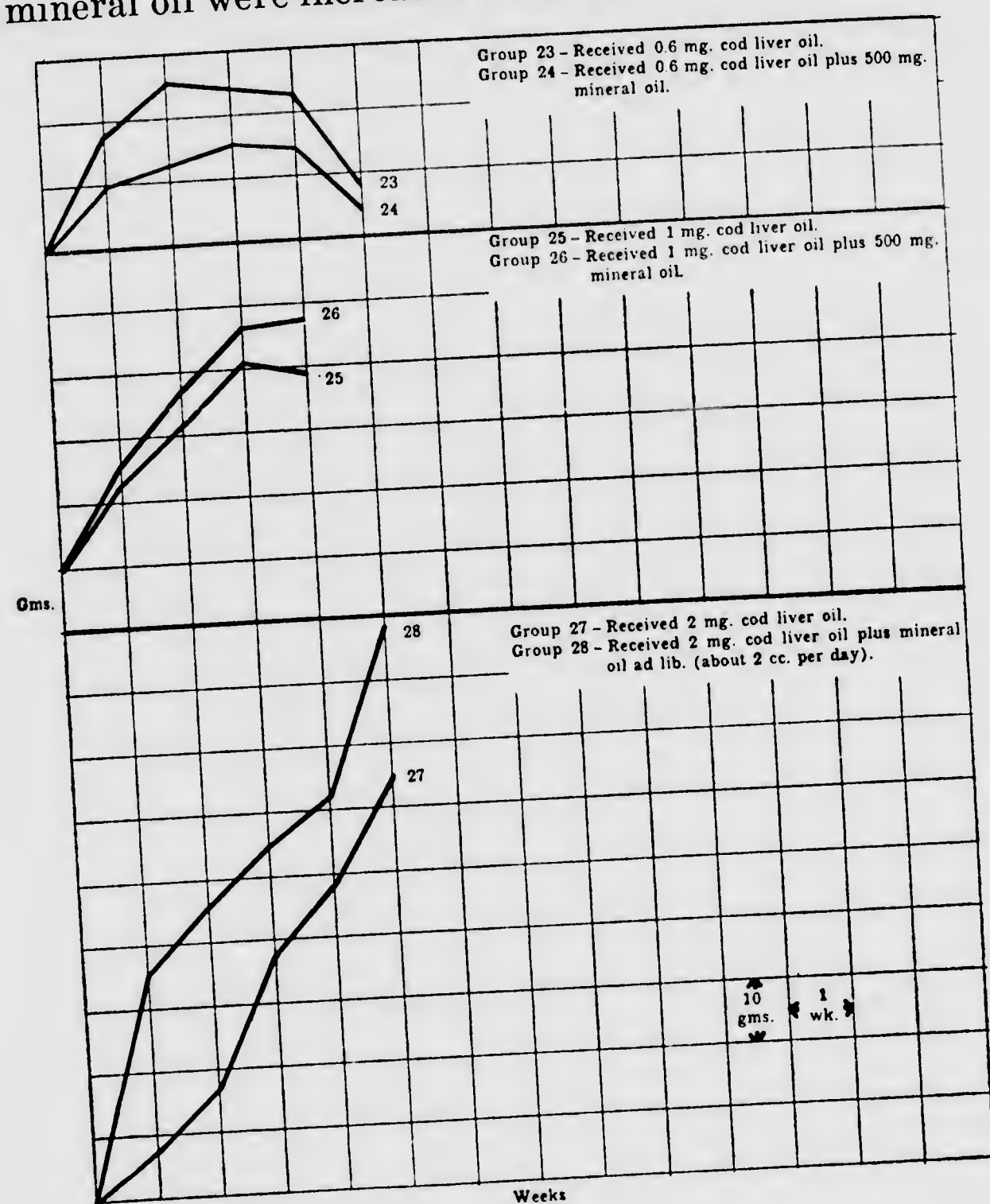


Chart 5

When a potent vitamin A concentrate was fed at a level approximating one Sherman unit (chart 6) in the presence and absence of varying amounts of mineral oil, no lowering of the vitamin A utilization could be noted.

It would appear, therefore, that carotene cannot be utilized when ingested in the presence of relatively small amounts of

mineral oil, while vitamin A (per se) is utilized quite efficiently when fed under similar conditions. Since hydroquinone had no tendency to improve utilization in the presence of mineral oil, it would appear that the lack of utilization is not due to oxidative destruction, which was postulated by Olcott and Mattill ('31).

All evidence seems to support the theory that mineral oil possesses a preferential solubility for carotene, preventing its absorption by removing it in solution from the digestive tract. In an endeavor to obtain additional evidence in support of this hypothesis, a second series of experiments was conducted.

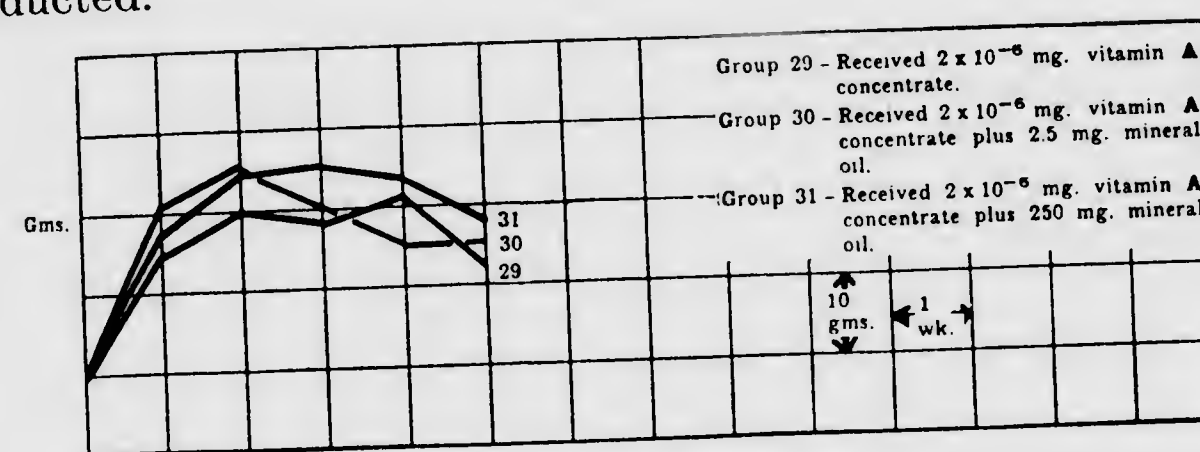


Chart 6

THE SECOND SERIES OF EXPERIMENTS

Prior to this work we had made repeated attempts to separate carotene and vitamin A from feces extracts obtained from rats which received these substances in mineral oil, with the view of re-feeding the carotene or vitamin A thus obtained, to prove, by biological response methods, that these substances are actually excreted in appreciable amounts. None of these attempts yielded satisfactory information owing to our inability to completely remove the mineral oil in order that it might not again vitiate results in the re-feeding period. Some encouraging data were obtained by adsorbing extracts in silica gel and similar adsorbants and by subsequent extraction of the adsorbant with various solvents (Zimmerman and Lachat, unpubl. data). None of these experiments was sufficiently quantitative for our purpose.

We also attempted to solve the problem by feeding varying amounts of carotene and vitamin A concentrate in the presence and absence of mineral oil, using 0.5 ml. of corn oil daily as in series 1. These feeding trials were of the prophylactic type in which the different groups of rats received carotene and vitamin A concentrate from the beginning of the experiment. Feces from these groups were collected daily and preserved under ether and the ether extracts were tested for carotene content with, a) the Lovibond tintometer, b) a colorimeter using a dichromate solution standardized against pure carotene, c) antimony trichloride color measurements, and, d) by spectroscopic measurements. These results were inconclusive, due to the pigmentation of the feces extracts with corn oil pigments and to other interfering substances. We also obtained evidence, by means of antimony trichloride tests and by rat assay methods, that our vitamin A concentrate was quite unstable in the new supply of corn oil.

As a result of these findings, we substituted ethyl laurate for corn oil and repeated the experiments, keeping the ratio of solvent to solute constant. The amounts of carotene, vitamin A concentrate and solvents are summarized in table 1.

Feces were collected daily from groups 32, 33, 34 and 35. These were preserved under petroleum ether during three periods of 10 days each. At the end of each 10-day period the feces were ground in petroleum ether and extracted with this solvent until no more color could be removed. These extracts were evaporated nearly to dryness and were made up to a volume of 10 ml. with redistilled chloroform.

Color values were obtained by comparing the color of the feces extracts with a standard dichromate solution in a colorimeter. No attempt was made to standardize this dichromate solution in terms of carotene, since we were interested primarily in showing whether or not a rough parallelism existed between increased carotene intake and increased carotene excretion.

The relative amounts of yellow pigment excreted during the increasing carotene intakes in the first 10-day period

are indicated graphically in chart 7. Data for the relative amounts of yellow pigment excreted during the second and third feces collection periods are omitted, since they were practically identical with those obtained during the first 10-day period.

TABLE 1

Group 32	No. of animal	1	2	3	4	5	6
Carotene in ethyl laurate	Amount of carotene	.001 mg.	.002 mg.	.003 mg.	.004 mg.	.005 mg.	.006 mg.
	Amount of solvent	.05 cc.	.10 cc.	.15 cc.	.20 cc.	.25 cc.	.30 cc.
Group 33	No. of animal	1	2	3	4	5	6
Carotene in mineral oil	Amount of carotene	.001 mg.	.002 mg.	.003 mg.	.004 mg.	.005 mg.	.006 mg.
	Amount of solvent	.05 cc.	.10 cc.	.15 cc.	.20 cc.	.25 cc.	.30 cc.
Group 34	No. of animal	1	2	3	4	5	6
A concentrate in ethyl laurate	Amount of A concentrate	.01 mg.	.02 mg.	.03 mg.	.04 mg.	.05 mg.	.06 mg.
	Amount of solvent	.05 cc.	.10 cc.	.15 cc.	.20 cc.	.25 cc.	.30 cc.
Group 35	No. of animal	1	2	3	4	5	6
A concentrate in mineral oil	Amount of A concentrate	.01 mg.	.02 mg.	.03 mg.	.04 mg.	.05 mg.	.06 mg.
	Amount of solvent	.05 cc.	.10 cc.	.15 cc.	.20 cc.	.25 cc.	.30 cc.

In addition to the colorimetric evidence submitted in chart 7, spectrophotometric determinations of carotene were made on two feces extracts¹ using a Konig-Martens spectrophotometer. The samples used were extracts from groups 32 and 33, which received 6 γ of carotene in ethyl laurate and mineral oil, respectively. The values obtained for $-\log_{10}$ of the transmittancy of 2 cm. were 0.41 for the extract from group 32 and 1.49 for group 33, which, according to the graph of

¹The writers are indebted to Dr. L. J. Briggs, director of the Bureau of Standards, Washington, D. C., for the spectrophotometric determinations of the carotene in these samples.

Schertz ('23), would correspond to 1 and 4 mg. of carotene per liter, respectively.

These values cannot be accepted as quantitative due to the fact that Schertz' measurements were made on carotene dis-

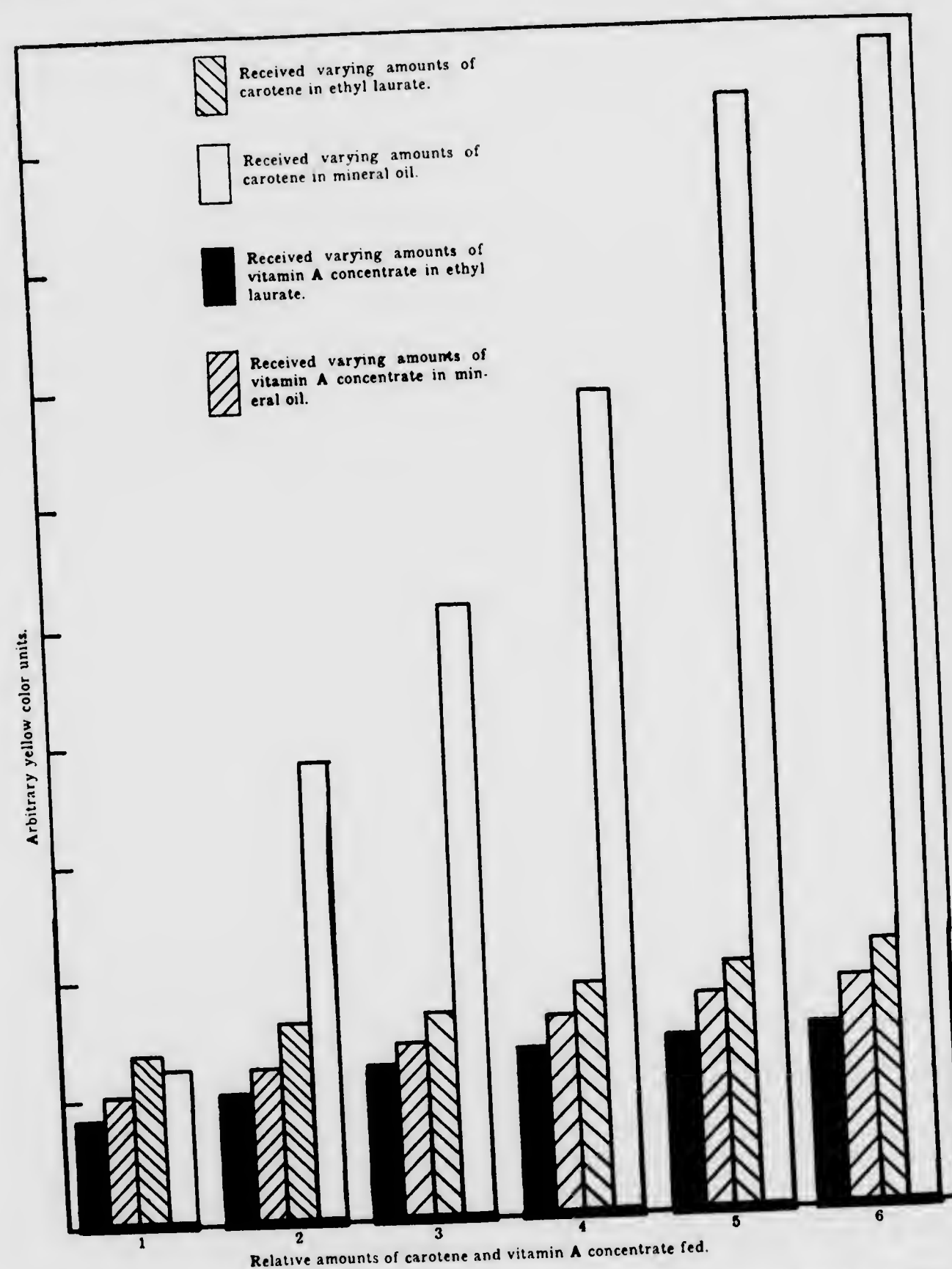


Chart 7 Showing relative intensity of feces extracts when rats received increasing amounts of carotene and vitamin A concentrate in ethyl laurate and mineral oil, respectively.

solved in ether, while our extracts were dissolved in chloroform. It is also certain that our extracts contained interfering substances. Since, however, the feces of both groups of animals were extracted under identical conditions, it would appear that the spectrophotometric data support the conclusion drawn from the color excretion data, viz., that the utilization of carotene is prevented by being excreted from the body in the unassimilated mineral oil.

Discussion of second series of experiments

The question as to the mechanism whereby mineral oil prevents the utilization of carotene is answered in the second series of experiments. Having eliminated, to our own satisfaction, the possibility of oxidative destruction of carotene by a pro-oxidant in mineral oil, we felt that the detrimental effect might be explained by a lack of carotene utilization due to loss from the body by being voided in the unabsorbed mineral oil.

This hypothesis is supported by data obtained when the yellow color was determined in feces from rats which received increasing amounts of carotene in ethyl laurate in the presence and absence of mineral oil. The curve of pigment excretion (group 33) indicates that color excretion was almost in direct proportion to the amount of carotene fed, indicating that the utilization of carotene was almost completely prevented by the mineral oil. Spectrophotometric determinations of carotene in the feces extracts also supported the above conclusion. This was indicated also in the growth response of the rats in groups 32 and 33. We noted also that the growth response in the ethyl laurate groups was somewhat inferior to that obtained in the corn oil groups. This has been observed by other workers.

CONCLUSIONS

1. Our previous results, showing that the vitamin A potency of butter fat is lowered, when fed at low levels in the presence of mineral oil, have been confirmed.

2. When butter fat was fed at higher levels in the presence of relatively small amounts of mineral oil, the deleterious effect of mineral oil was less marked, although the effect could still be noted.

3. The vitamin A potency of cod liver oil and of a cod liver oil concentrate was not adversely affected by the presence of mineral oil, which undoubtedly explains the less harmful effects of mineral oil on butter fat at the higher feeding levels, since a part of the potency of butter fat is due undoubtedly to vitamin A per se, while only a part of the potency can be ascribed to carotene.

4. The harmful effect of mineral oil can be explained on the basis of carotene excretion from the body in the unabsorbed mineral oil. This hypothesis is supported by the fact that yellow pigment excretion (when mineral oil is fed) is roughly proportional to the carotene ingested. This is not true when carotene is fed in the absence of mineral oil. Spectrophotometric determinations of carotene in the feces extracts also support this conclusion.

5. The hypothesis is advanced that the hydrocarbons of the unassimilated mineral oil possess a greater solvent effect on the hydrocarbon carotene than is possessed by the lipids of the intestinal juices, thereby preventing intestinal absorption of carotene. Conversely, it is suggested that the lipids and sterols of the digestive juices possess a preferential solvent effect on the sterol vitamin A, thereby promoting utilization by removing this vitamin from the unassimilated mineral oil.

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SOME EFFECTS OF THE COMPOSITION OF THE DIET ON THE VITAMIN B AND THE VITAMIN G REQUIREMENT OF THE GROWING RAT ¹

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EIGHT FIGURES

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A previous communication from this laboratory (Guerrant and Dutcher, '33) contained data which suggested that the fiber content of the diet might have a marked effect on the amount of vitamin B complex required by the rat. A review of the available literature at that time revealed that other investigators had made similar observations concerning some of the other constituents of the basal diets commonly employed in vitamin B and vitamin G techniques. Most of these observations, however, had been made in connection with the protein and fat content of the diet.

Drummond, Crowden and Hill ('22), Reader and Drummond ('25), Hartwell ('25), Reader and Drummond ('26), Hassen and Drummond ('27), Sherman and Gloy ('27), Hartwell ('28) and Guha ('31) had reported observations concerning various relations of the B complex vitamins to protein utilization. The role played by fats in this connection had been studied by Evans and Lepkovsky ('28, '29) and Guha ('31).

It was difficult to account for the variations in the conclusions drawn by the above investigators. It seemed possible,

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however, that a part, at least, of the lack of uniformity of results might have been due to the fact that vitamin B was not recognized as a complex at the time when some of the experiments were conducted. This fact and, in addition, the wide variations in the composition of basal diets employed by different laboratories in their vitamin B and vitamin G techniques, led us to believe that a study of the effects of variations in the composition of the diet on the vitamin B and the vitamin G requirement of the rat could be made with profit.

During the course of this investigation, other communications have been published by Sherman and Derbigny ('32), Evans and Lepkovsky ('32 a, '32 b), Lecoq ('32 a, '32 b, '32 c, '33), Lecoq and Savare ('33), Prunty and Roscoe ('33), Hogan and Pilcher ('33), Francis, Smith and Mendel ('33), Gregory and Drummond ('32), and by Patras and Templeton ('33), which have a bearing on this subject.

The data herein reported give some of the results of a series of experiments carried out during the past 2 years in an effort to gain some insight into the influence of the composition of the diet on the vitamin B and the vitamin G requirement of the growing rat.

EXPERIMENTAL

The general plan followed was to feed groups of rats on diets of varied composition, but deficient in both vitamins B and G until early symptoms of vitamin B deficiency appeared; then to supplement such diets with daily dosages of vitamin B concentrate, while continuing the experiment for several weeks until some manifestations of vitamin G deficiency became apparent, at which time further supplementation was effected by daily additions of autoclaved yeast and the animals were continued under observation for several additional weeks.

Both piebald and albino rats were used in the investigation. These animals were placed on experiment in groups of from 5 to 12 animals each, care being taken in their group distribution in order to minimize both litter and sex variations.

Groups of rats, 20 to 21 days old and weighing 39 to 46 gm. each, were placed and maintained in individual cages provided with raised screen grids, and were fed liberal quantities of the diets under consideration. Clean distilled water was kept before the animals at all times. Small additions of iodine were given at weekly intervals. Weekly records were made of the quantity of food consumed, the change in body weight, and the general appearance of each animal. After a preliminary depletion period of 21 days on such diets, each animal was given a measured daily dosage of the vitamin B concentrate during the next 42 to 56 days as a supplement to the diet. At the end of this period, the diet of each animal was further supplemented by weighed daily allotments of autoclaved yeast and the animal was continued under observation for at least 6 additional weeks. Thus all animals which survived the experimental period had existed for 21 days on a diet deficient in both vitamins B and G, followed by 42 to 56 days on the same diet supplemented by daily allotments of vitamin B, and finally for at least 42 days on this diet supplemented by both vitamins B and G.

The vitamin B supplement used in these studies was prepared from dried brewer's yeast. The dry yeast was extracted by percolation with 95 per cent alcohol which had been acidified with concentrated hydrochloric acid (5 ml. of acid per liter of alcohol) as long as the percolate remained colored. The combined percolate was concentrated under reduced pressure until the residue assumed a sirupy consistency. This concentrate was placed in a separatory funnel, an equal volume of water was added, and the mixture was shaken with three successive portions of ether (100 ml. of ether for each kilogram of yeast extracted) in order to remove most of the fatty materials. After partially neutralizing the excess acid with sodium bicarbonate, the concentrate was again reduced to a sirupy consistency by vacuum distillation, and was maintained at a sub-zero temperature for 24 hours. The insoluble materials were filtered off rapidly by means of suction, and sufficient 95 per cent alcohol was

added to make a volume such that 1 ml. of the concentrate represented 10 gm. of the original yeast. This concentrate was kept in the refrigerator during the course of the investigation. A daily dose of 0.1 ml. of this solution was used as a source of vitamin B. Previous tests had shown this quantity to be sufficient to stimulate appreciable growth, when fed to rats receiving a diet complete in other respects.

The vitamin G supplement was prepared from baker's yeast by moistening ^{the} dry yeast with a 10 per cent solution of sodium bicarbonate, and then autoclaving the moist mixture for 6 hours at 15 pounds pressure. The autoclaved yeast was later dried and ground to a fine powder. A daily dose of 0.3 gm. of this product was found to supply adequate vitamin G when fed as the only source of this vitamin.

The control diet was similar in composition to that which had previously been used in this laboratory in vitamin B and vitamin G studies. It consisted (in parts per 100) of washed casein 18, salt mixture (McCollum 185) ('18) 4, agar 2, sucrose 20, cod liver oil 2, butter fat 3, and dextrin 51. A diet of this composition was chosen, because it offered possibilities of quite wide variations in its constituents at the expense of the sucrose. This quality was highly desirable, since sucrose, a fairly pure and uniform source of carbohydrate, was selected as the variable compensator in the variations of the other constituents of the diet. This diet alone, and in combination with daily allotments of the vitamin B concentrate, the vitamin G fraction, and a combination of these two preparations, was fed to groups of depleted rats at intervals during the progress of the investigation. These tests were carried out in order to test the adequacy of the daily dosage of the two vitamins, and to show that definite growth responses could be produced by feeding rats a diet of this composition, when it is properly supplemented.

Using a diet of this composition as a basis, five series of diets, in each series of which one of the above ingredients was varied systematically within practical limits, were fed to respective groups of rats during a three-phase experimental period throughout which the animals received 1) neither vita-

min B nor vitamin G, 2) vitamin B, and 3) vitamins B and G. In addition, a sixth series (of four diets), in which 2, 4, 6, and 8 per cent of CellU flour were used as sources of roughages instead of agar, was similarly tested. In order to conserve space, the number and composition of the various diets used are presented in table 1.

The components of the various diets were of the quality that is most commonly used in nutritional work of this type. The casein was a good grade South American product. It was extracted with both acidulated water and 95 per cent alcohol until practically free of the vitamin B complex. All ingredients used in the salt mixtures were of the C.P. grade except the ferric citrate and the calcium lactate. These salts were of the U.S.P. grade. The agar was a U.S.P. powder obtained from a reliable chemical supply company. Refined sucrose of the usual commercial grade was used. The Crisco was purchased from a local grocery. The cod liver oil was the E. L. Patch medicinal product. The butter fat was prepared in the usual manner from fresh creamery butter purchased from the college creamery. The dextrin was prepared from commercial cornstarch by moistening the starch with a dilute solution of citric acid and autoclaving for 4 hours at 15 pounds pressure. The CellU flour was a highly purified form of cellulose furnished through the courtesy of the Chicago Dietetic Supply House, Inc. All diets were made up at weekly intervals and were stored in an electric refrigerator.

DATA

While close observation was maintained upon all animals during the course of the investigation and a fairly complete record was kept at all times, it is believed that the various differences in growth responses manifested by the several groups of rats receiving the different diets are the most accurate indices of the rat's requirement for vitamin B and for vitamin G while subsisting on these various dietaries. For this reason and also to conserve space, the data obtained in the several series of experiments have been condensed and presented in table 2 and in figures 1 to 8, inclusive.

TABLE 1
Showing the number and the composition of the various diets used

DIET NO.	CRISCO	SALTS	CASEIN	AGAR AGAR	CELLU FLOUR	DEXTRIN	SUCROSE	COD LIVER OIL	BUTTER FAT
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
306 ¹	0	4	18	2	0	51	20	2	3
324	0	4	18	2	0	51	25	0 ²	0 ²
319 ¹	0	4	18	2	0	51	15	2	3
320	5	4	18	2	0	51	10	2	3
321	10	4	18	2	0	51	5	2	3
322	15	4	18	2	0	51	0	2	3
323	20	4	18	2	0	51	23	2	3
328	0	1	18	2	0	51	22	2	3
325	0	2	18	2	0	51	20	2	3
329 ¹	0	4	18	2	0	51	18	2	3
326	0	6	18	2	0	51	16	2	3
327	0	8	18	2	0	51	14	2	3
330	0	10	18	2	0	51	30	2	3
331	0	4	8	2	0	51	26	2	3
332	0	4	12	2	0	51	22	2	3
333	0	4	16	2	0	51	18	2	3
334	0	4	20	2	0	51	14	2	3
335	0	4	24	2	0	51	10	2	3
336	0	4	28	2	0	51	22	2	3
337	0	4	18	0	0	51	20	2	3
338 ¹	0	4	18	2	0	51	18	2	3
339	0	4	18	4	0	51	16	2	3
340	0	4	18	6	0	51	14	2	3
341	0	4	18	8	0	51	12	2	3
342	0	4	18	10	0	51	20	2	3
345	0	4	18	0	4	51	18	2	3
346	0	4	18	0	6	51	16	2	3
347	0	4	18	0	8	51	14	2	3
348	0	4	18	0	8	51	71	2	3
353	0	4	18	2	0	11	60	2	3
352	0	4	18	2	0	31	40	2	3
351	0	4	18	2	0	51	20	2	3
350 ¹	0	4	18	2	0	71	0	2	3
349	0	4	18	2	0	71	0	2	3

¹ These diets all had the same composition but were designated under different numbers, since they were used as control diets in different series and at different times.

² Crystalline carotene and 10,000 D viosterol were dissolved in petroleum ether and added to the basal diet at weekly intervals in sufficient quantities to furnish 10 Sherman units of vitamin A and 10 A.D.M.A. units of vitamin D for each gram of food.

DIET NO.	VARIABLE CONSTITUENT	PER CENT VARIABLE CONSTITUENT PRESENT	NUMBER OF ANIMALS CONSIDERED	AVERAGE INITIAL WEIGHT	GAIN DURING DEPLETION PERIOD	GAIN DURING B PERIOD	GAIN DURING B + G PERIOD	TOTAL GAIN IN WEIGHT	WEEKLY FOOD INTAKE DURING DEPLETION PERIOD	WEEKLY FOOD INTAKE DURING B PERIOD	WEEKLY FOOD INTAKE DURING B + G PERIOD	APPROXIMATE CALORIC INTAKE INCREASE IN WEIGHT ¹	During the B + G period	During the B period
306	Unsupplemented		9	42	7	17	..	7	23	21	..	42
306	Vitamin B concentrate		18	41	7	17	..	24	23	21	..	42	39	..
306	Autoclaved yeast		10	41	8	..	96	17	23	..	17	33
306	Vitamins B and G		9	42	6	102	23	..	32	46
324	Fat	0	5	40	-2	14	34	46	16	18	24	..	37	22
319	Fat	5	5	41	3	13	35	51	17	19	26	69	47	26
320	Fat	10	5	42	4	8	44	56	19	16	25	60	50	21
321	Fat	15	5	40	4	12	50	66	19	17	27	64	51	21
322	Fat	20	5	41	4	14	59	77	18	18	31	64	49	21
323	Fat	25	5	40	6	11	48	65	17	16	25	53	58	23
328	Inorganic	1	5	43	-1	20	46	65	20	24	31	..	30	21
325	Inorganic	2	6	42	10	17	34	61	22	23	28	27	33	25
329	Inorganic	4	6	41	9	11	26	46	22	22	24	29	48	28
326	Inorganic	6	6	41	11	8	25	44	21	17	23	23	50	28
327	Inorganic	8	5	42	10	5	23	38	21	16	22	24	74	30
330	Inorganic	10	6	43	6	0	15	21	21	16	22	40	44	44
331	Casein	8	7	41	-3	8	37	42	21	24	27	..	96	19
332	Casein	12	10	41	4	11	42	57	22	27	29	66	79	18
333	Casein	16	9	40	7	13	36	56	22	26	29	64	64	21
334	Casein	20	10	40	8	12	40	60	23	25	28	67	67	18
335	Casein	24	10	41	7	14	38	59	22	24	29	55	55	20
336	Casein	28	6	40	4	16	29	49	20	23	28	46	46	25
337	Agar	0	10	40	4	17	34	55	24	23	29	72	33	21
338	Agar	2	10	41	7	16	36	59	24	25	31	38	38	22
339	Agar	4	9	41	10	22	33	65	26	27	34	43	43	22
340	Agar	6	10	42	13	19	38	70	29	29	35	30	30	26
341	Agar	8	10	41	17	22	33	72	26	28	38	31	31	29
342	Agar	10	10	42	16	23	37	76	25	28	37	19	28	25
345	Cellu flour	2	6	40	7	20	32	54	22	26	31	38	31	24
346	Cellu flour	4	6	41	13	20	33	66	22	27	34	20	33	27
347	Cellu flour	6	6	41	13	22	33	68	23	26	38	21	28	29
348	Cellu flour	8	6	42	19	25	30	74	25	32	36	16	31	30
353	Dextrin	0	4	42	-7	2	21	16	19	15	18	..	181	24
352	Dextrin	11	5	43	-6	2	19	15	19	16	19	..	192	25
351	Dextrin	31	6	41	-3	2	36	33	20	17	23	..	136	17
350	Dextrin	51	6	42	-3	8	41	46	21	19	27	..	57	19
349	Dextrin	71	6	42	-5	14	46	65	23	22	31	55	38	17

¹ Calculated on the basis that protein = 4 cal. per gram, carbohydrate = 4 cal. per gram, fat = 9 cal. per gram, and that casein and yeast are 100 per cent protein.

DISCUSSION

In compiling the above data, records of individual animals which departed abnormally from the group averages were omitted, and only those animals were considered which reacted more or less uniformly as a group. The number of animals which failed to meet this requirement was not large, but did appear to be greater in certain experimental groups. In most cases, these abnormal responses seemed to have followed an excessive period of depletion during the first stage of the experiment. Most of these animals had manifested marked symptoms of beriberi before the end of the 21-day depletion period, and did not make the immediate characteristic response when the daily allotment of the vitamin B concentrate was added. It appears most probable that more uniform growth responses would have been obtained in certain groups of rats had this depletion period been reduced by several days. There is, on the other hand, some evidence which indicates that groups of rats receiving certain diets, could have withstood a more prolonged depletion period without serious consequence.

During the course of the investigation, a total of forty-six animals were used in testing the basal diet and the effectiveness of the vitamin B and the vitamin G fractions in supplementing this diet. The data obtained (fig. 1) show that, a) all rats that received the basal diet unsupplemented died between the twenty-ninth and the fifty-ninth days, b) seventeen of the eighteen rats that received the basal diet supplemented by the vitamin B concentrate lived through the 8-week experimental period, c) nine of the ten animals that received the basal diet supplemented with the vitamin G fraction died before the end of the experiment, and d) those animals that received the basal diet supplemented by both the vitamin B concentrate and the vitamin G fraction made an average gain of 11 gm. per week during the 8-week period.

It was interesting to note in connection with the control diet that, while the composition of the diet remained unchanged from beginning to end, the first animals to die mani-

festated no apparent symptoms of beriberi other than an unsteady gait, and when found dead they were usually clutched to the cage by paws and teeth. Those animals which survived longer, however, manifested very characteristic symptoms several days previous to death. These facts, together with observations made in connection with other experiments, suggest that some relation must exist between the age of the rat and the type of vitamin B deficiency symptoms manifested.

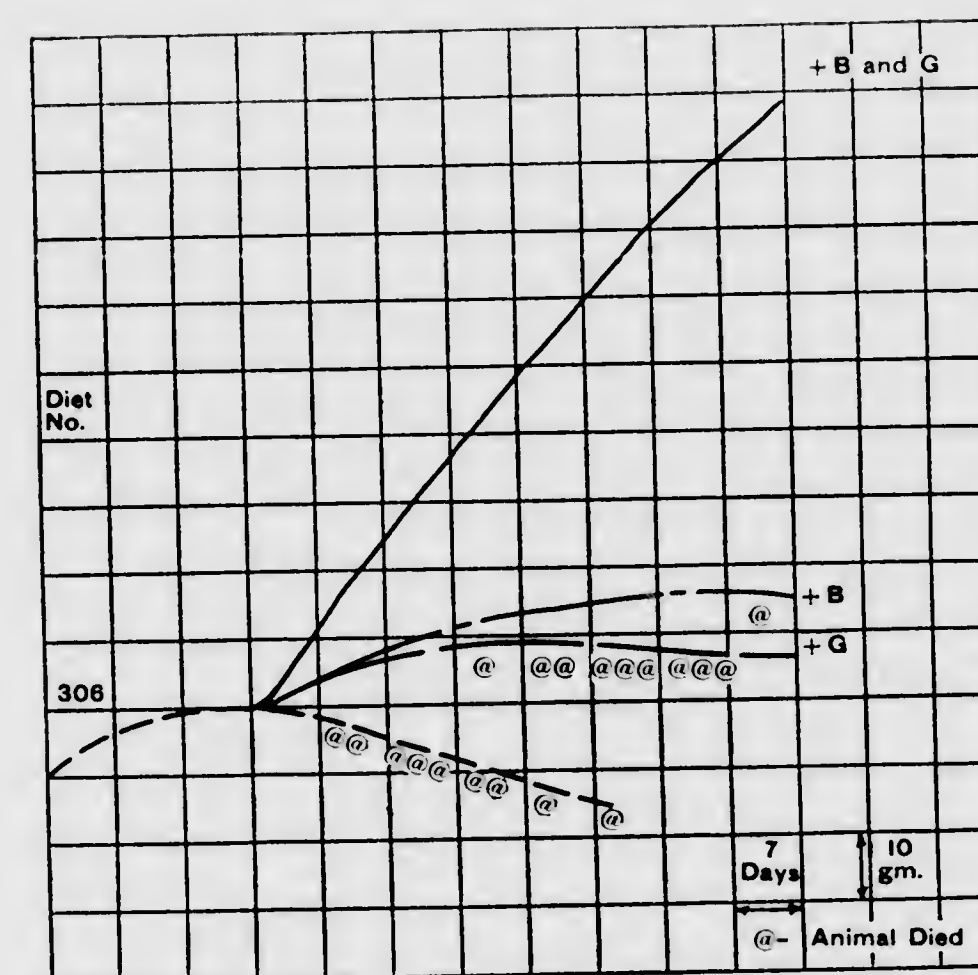


Fig. 1 Showing the effect of supplementing the basal diet (306) with 0.1 ml. of vitamin B concentrate and 0.3 gm. of the vitamin G fraction, separately and in combination.

In a consideration of the data obtained from the groups of rats receiving the diets comprising the fat series (fig. 2), several interesting trends become apparent and seemed to justify some consideration. Diet 324 had been made practically fat free by extracting the properly combined ingredients with ethyl ether for 16 hours in a continuous extractor. Sufficient crystalline carotene and 10,000 D viosterol were added to the ether-free diet to furnish 10 units each of vitamin A

and vitamin D for each gram of food. By using highly potent sources of these vitamins, the fat content of the diet was maintained at the lowest practical level. By increasing the fat content of this diet first by replacing a portion of the sucrose by cod liver oil and butter fat in order to supply the fat soluble vitamins, and then by further replacement of the

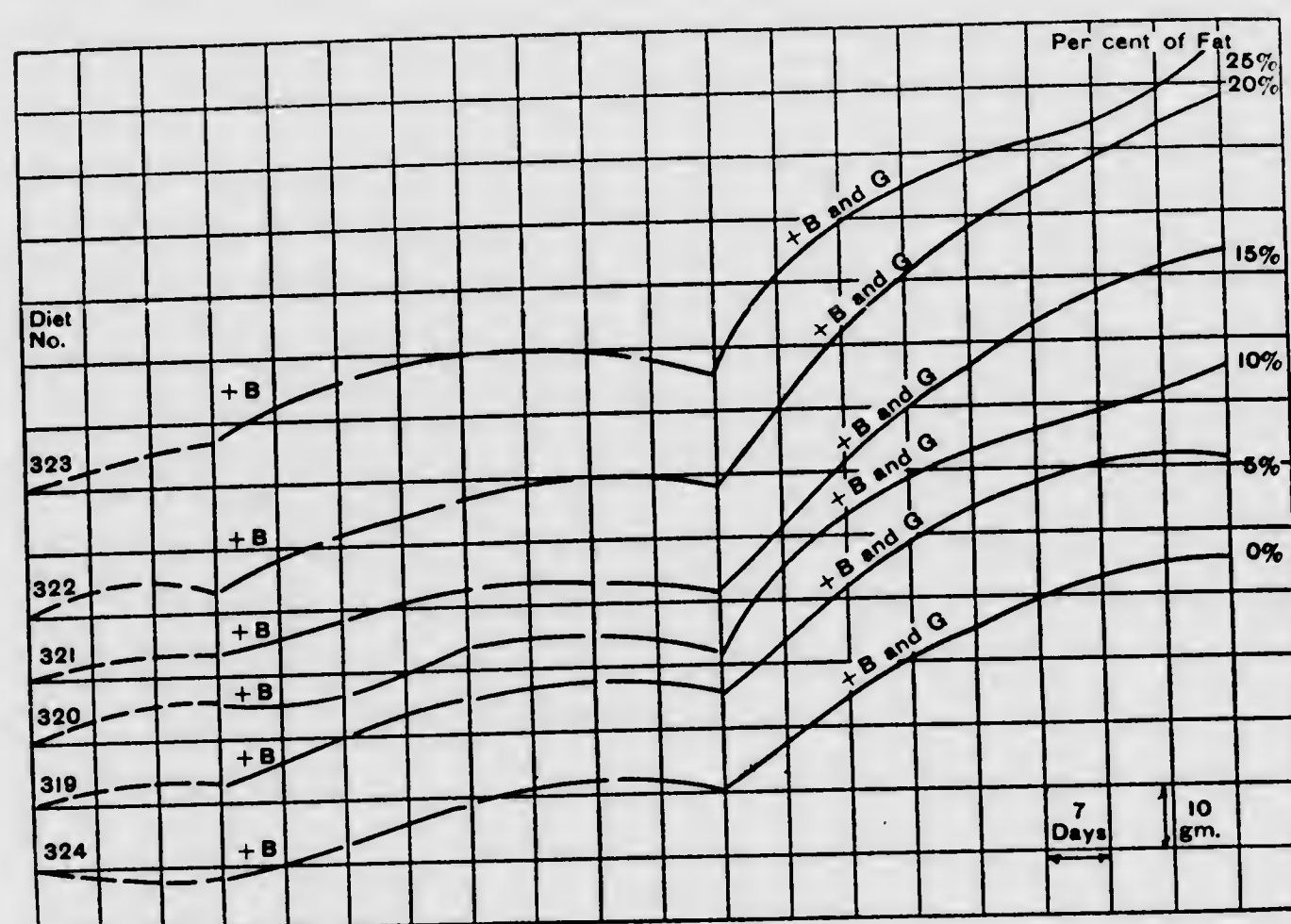


Fig. 2 Showing the average growth responses made by groups of rats receiving 1) vitamin B complex deficient diets of varying fat content; 2) these diets supplemented with daily allotments of 0.1 ml. of the vitamin B concentrate, and, 3) these diets supplemented daily with both 0.1 ml. of the vitamin B concentrate and 0.3 gm. of the vitamin G fraction.

sucrose with 5, 10, 15 and 20 per cent of Crisco, a series of diets were made up that ranged in fat content from 0 to 25 per cent.

The data obtained through feeding this series of diets to groups of young rats emphasizes three points worthy of note. a) Rats receiving diets containing appreciable quantities of fat (Crisco) were more difficult to deplete of their vitamin B stores than similar animals receiving diets of low fat con-

tent. b) The data do not indicate that the fat content of the diet bears any relation to the rat's requirement for vitamin G. c) When restricted daily allotments of both vitamins B and G were fed to rats, greater growth rates were obtained from those animals receiving diets containing from 15 to 20 per cent of fat than from similar animals receiving corresponding diets of lower fat content.

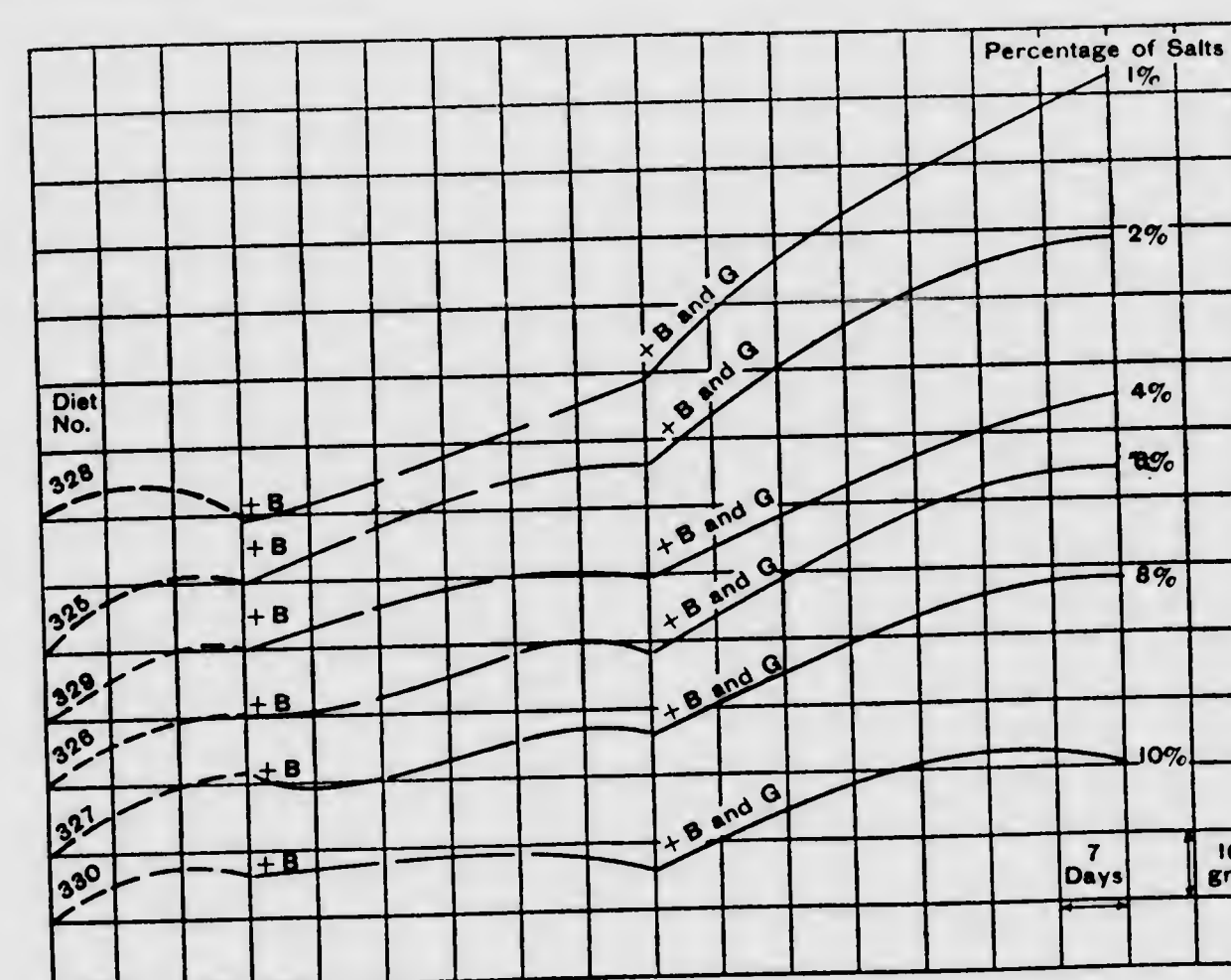


Fig. 3 Showing the average growth responses made by groups of rats receiving, 1) vitamin B complex deficient diets of varying salts content; 2) these diets supplemented with daily dosages of 0.1 ml. of the vitamin B concentrate, and, 3) these diets supplemented with daily dosages of both 0.1 ml. of the vitamin B concentrate and 0.3 gm. of the vitamin G fraction.

The results obtained with the series of diets in which the salt mixture was the variable constituent under consideration likewise revealed some points of interest (fig. 3). The group of rats which received diet 330 containing 10 per cent of the salt mixture made less than the usual growth response during the 3-week depletion period. In this case the vitamin B concentrate appeared to have little or no supplementing

value. When this diet was further supplemented by autoclaved yeast, the animals grew at a very low rate during the next 5 weeks. During the last 2 weeks that this group of animals was under observation, increased evidence of nutritional failure became apparent. It was highly improbable if any would have lived for 2 additional weeks had the experimental period been extended.

The group of rats receiving diet 327, which contains 8 per cent of the salt mixture, presented a better nutritional picture throughout the experimental period than did those animals receiving the diet containing 10 per cent of this constituent. In fact, as the salt content of the various diets composing the series was decreased through 8, 6, 4 and 2 per cent (diets 327, 326, 329 and 325) more favorable growth responses were obtained. This was also true with diet 328, which contained only 1 per cent of the salt mixture. Frequent observations, however, of the various animals composing the several groups of this series led us to believe that the results which were obtained with this diet are not entirely free of an unsuspected experimental error. At the end of the third week the animals of this group had shown a definite loss in weight, but presented no evidence of beriberi. When the diet was supplemented by vitamin B concentrate, the animals made an immediate growth response. Close observations at this time revealed all animals of the group to be consuming their excreta, especially the urine. This tendency was evidently acquired about the end of the depletion period and was continued throughout the experiment. Consequently, the data obtained on diet 328 are difficult to evaluate in terms of either vitamins B and G or salts intake. The practice of coprophagy was seldom observed among the other groups of animals comprising this series. It is believed, therefore, that the practice in this particular group was an attempt on the part of the experimental animals to maintain a favorable mineral balance while receiving a diet inadequate in inorganic constituents.

When a series of diets, in which the protein constituent (casein) was varied in 4 per cent intervals from 8 to 28

per cent, were fed to groups of rats receiving controlled daily allotments of vitamins B and G, under comparable conditions, no startling differences in growth responses were obtained (fig. 4). The group of rats which received diet 331 containing 8 per cent of casein lost weight during the first 3 weeks that they were on experiment. While none of the animals

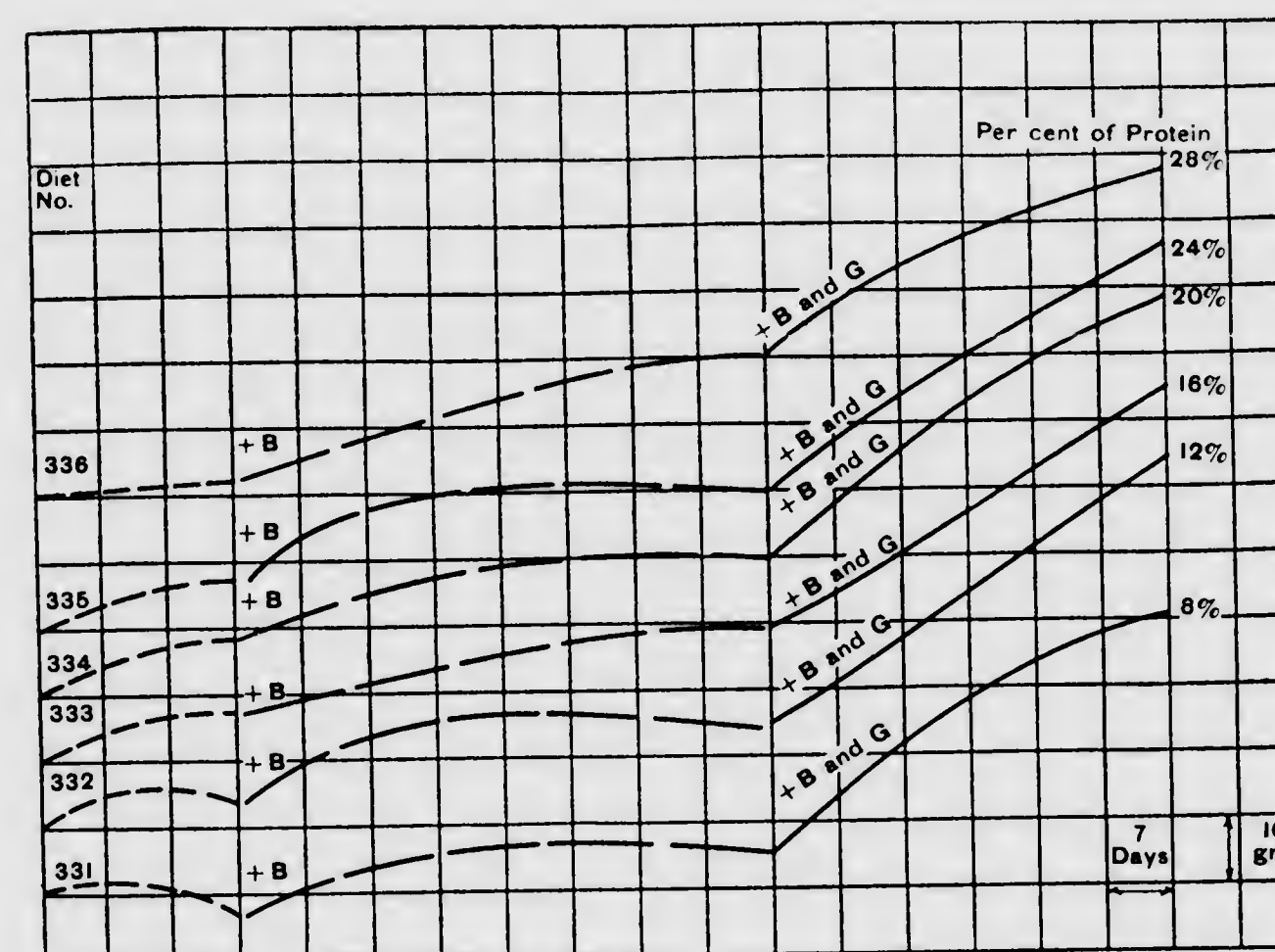


Fig. 4 Showing the average growth responses made by groups of rats receiving, 1) vitamin B complex deficient diets of varying protein content; 2) these diets supplemented daily with 0.1 ml. of the vitamin B concentrate, and, 3) these diets supplemented by both 0.1 ml. of the vitamin B concentrate and 0.3 gm. of the vitamin G fraction.

of this group died from beriberi during this time, some of them did exhibit marked paralytic symptoms. Later, three of the ten animals comprising the group were found to be practicing coprophagy; consequently they were removed from the experiment. When a daily allotment of the vitamin B concentrate was given to each animal of this group, a slight gain in weight amounting to 10 gm. resulted during the next 5 weeks, part of which was subsequently lost. The animals

of this group, after being depleted for 3 weeks, and then receiving vitamin B for 8 weeks, did not show any severe symptoms of vitamin G deficiency. There was the usual roughness of hair, scaly feet, and some local depilation of head and body. When the diet was further supplemented with autoclaved yeast, each animal of the group made a very definite growth response and improved markedly in both general appearance and well-being. In this case, it is difficult to determine whether all of this increased growth was due to vitamin G or whether part of it should be attributed to the supplementing effect of the protein added in the form of autoclaved yeast.

When the diet contained 12, 16, 20 or 24 per cent of casein, respectively, as in diets 332, 333, 334 and 335, a definite improvement in growth resulted in all three phases of the experiment when compared to the results obtained on the lower protein diet (331). The group of rats which received diet 336, containing 28 per cent of casein, made only a very slight gain in weight during the first 3 weeks of the experiment. At first there were some indications that this diet was somewhat unpalatable to the young rats. When this diet was supplemented by the vitamin B concentrate, the animals grew at an average rate of 2.5 gm. per week for the next 8 weeks. At this time, the animals of this group had made about the same increase in weight as the groups receiving diets 333, 334 and 335. On further supplementing diet 336 with autoclaved yeast, these animals increased their average rate of growth to 5 gm. per week for the next 6 weeks, which was less than the growth rates of several other groups during this same period.

The data obtained through feeding rats this series of diets indicate that, within the limits of the usual experimental diet, slight difference in protein has no measurable effect upon the rat's requirement for vitamins B and G. When the protein content of the diets is increased or decreased beyond certain limits, other complications arise which make it more difficult to interpret the experimental results obtained.

When a series of six diets, in which agar was the variable constituent, were fed to respective groups of rats, some noticeable differences were observed in the resultant growth responses (fig. 5). The ten animals that received diet 337, which contained no agar, made a slight gain in weight during the first 2 weeks of the depletion period, but part of this gain was later lost during the third and last week of the period.

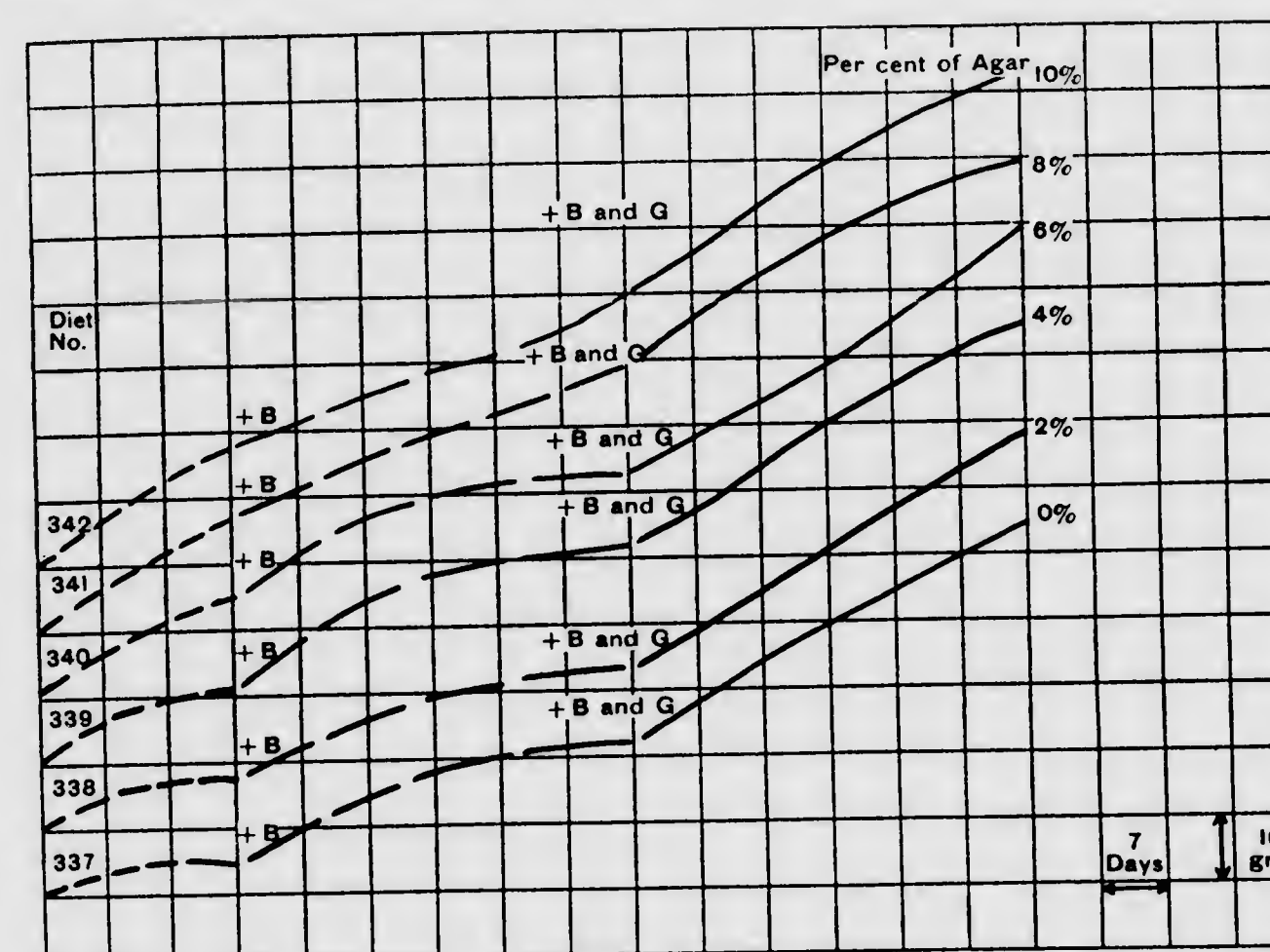


Fig. 5 Showing the average growth responses made by groups of rats receiving, 1) vitamin B complex deficient diets of varying agar content, 2) these diets supplemented with 0.1 ml. daily allotments of the vitamin B concentrate, and, 3) these diets supplemented with both 0.1 ml. of the vitamin B concentrate and 0.3 gm. of the vitamin G fraction.

At this time all animals of the group appeared to be in an unthrifty condition and some of them showed definite paralytic symptoms. On supplementing this diet with the vitamin B concentrate, all animals reassumed growth for the next 5 weeks. Ruffled fur and retarded growths appeared to be the only visible external symptoms of vitamin G deficiency. On further supplementing the diet with autoclaved yeast, all animals of the group made a uniform and uneventful growth of 5 gm. per week for the next 6 weeks.

When the diet contained 2 per cent of agar (diet 338), the growth response, in general, was very similar to that obtained on the agar-free diet. The only noticeable difference was a smaller loss in weight during the depletion period and fewer symptoms of beriberi during this time. As the agar content of the diets was further increased in 2 per cent intervals, as in diets 339, 340, 341 and 342, marked differences appeared in the responses made by the respective groups of rats during the depletion period. The group of rats receiving a diet containing 10 per cent of agar grew at approximately the same rate as that group which received a diet containing only 8 per cent of this constituent. Those animals which received the diets of higher agar content (6, 8 and 10 per cent) were not only free of beriberi at the end of the 3-week depletion period, but appeared to be in a fair state of nutrition. In spite of marked differences in the growth responses and in the general appearance of the six groups of animals at the end of the 3 weeks, all groups responded quite uniformly when these diets were supplemented, first by the vitamin B concentrate and later by both the vitamin B concentrate and the vitamin G fraction. The results indicate quite conclusively that the uniform addition of vitamins B and G became less effective in increasing the growth rate of the animals as the diets became richer in agar content. This difference in supplementing value was more marked in the case of the vitamin B concentrate.

The above data in themselves were not sufficient to justify the postulation of a specific mechanism by which the vitamin B and the vitamin G requirement of the rat could be affected by the quantity of agar incorporated in the diet. A number of different possibilities suggested themselves, but none appeared tenable without further data. To us the above data appeared to be open to criticism on the ground that an impure or an unrefined agar had been used in the various diets. We anticipated considerable difficulty in attempting to free the agar from impurities by any mode of extraction with vitamin solvents. A more logical plan appeared to be a repetition

of at least part of the experiments involving the agar-containing diets, using CellU flour instead of agar as a source of roughage.

In the CellU flour series, only four diets were fed. Diets 345, 346, 347 and 348 (fig. 6) contained 2, 4, 6 and 8 per cent, respectively, of CellU flour and in other respects were identical in composition to diets 338, 339, 340 and 341 of the agar

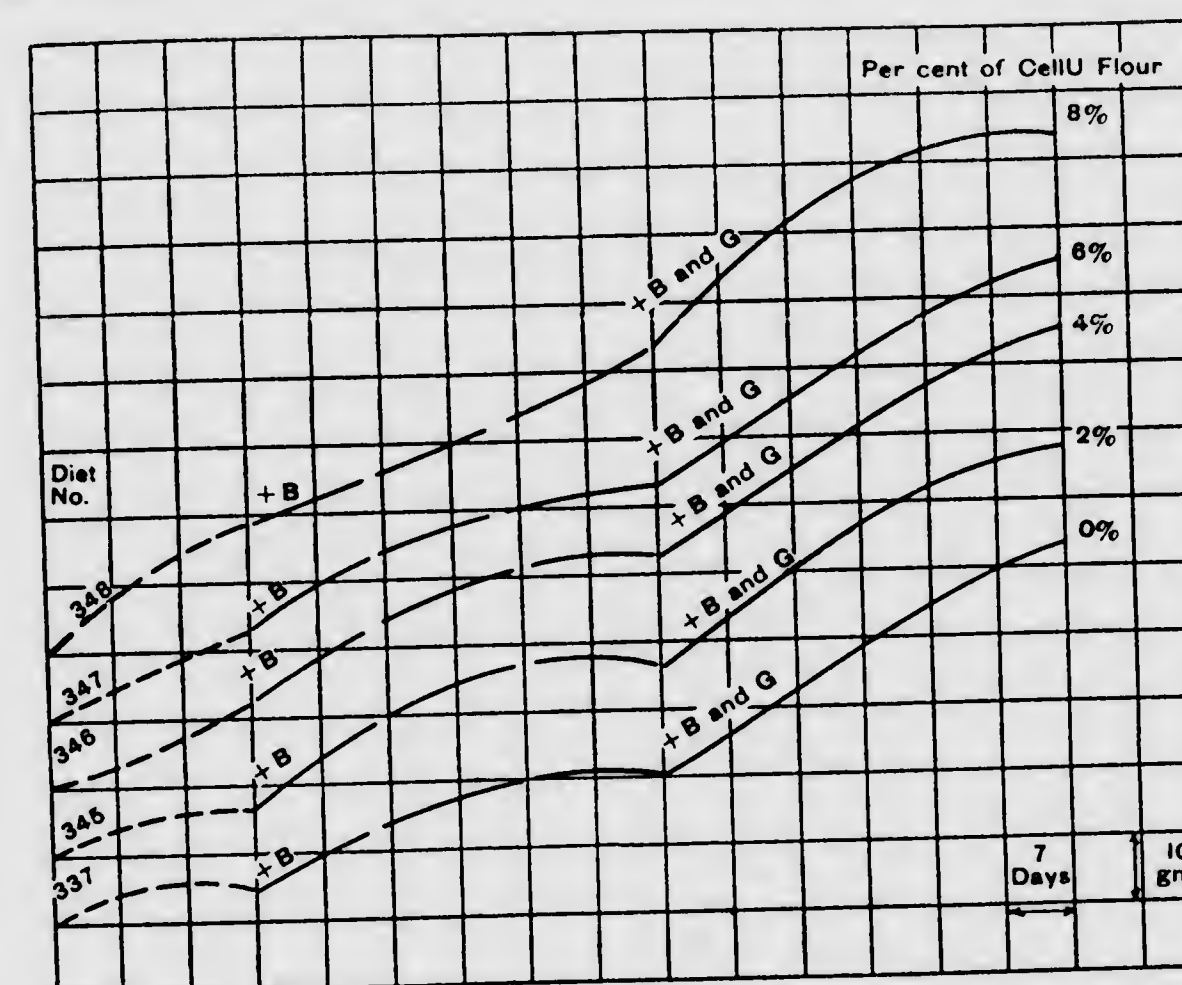


Fig. 6 Showing the average growth responses made by groups of rats receiving, 1) vitamin B complex deficient diets of varying CellU flour content, 2) these diets supplemented by daily additions of 0.1 ml. of the vitamin B concentrate, and, 3) these diets supplemented by daily additions of both 0.1 ml. of vitamin B concentrate and 0.3 gm. of the vitamin G fraction.

series. The test with diet 337 was not repeated at this time. The growth curve presented is the same as presented in figure 5. Four groups of rats of six animals each were fed the CellU flour diets under conditions as nearly identical as possible to that maintained in the agar series. A comparison of figures 5 and 6 will reveal the striking similarity in the two series of results obtained. The nutritional pictures presented by the two groups of rats, one group of which re-

ceived a diet containing a definite percentage of agar, while the other group received a diet containing an equal percentage of CellU flour, were so similar in all respects that a rediscussion appears unnecessary at this time.

While no satisfactory explanation can be offered at this time relative to the physiological mechanism by which the fiber (agar and CellU flour) content of the diet influences the rat's requirement for vitamin B and also vitamin G (but to a less degree), it is hoped that experiments in progress at the present time may shed some light upon the subject.

Unpublished experimental data obtained in this laboratory showed that marked differences in the growth responses of groups of rats receiving identical quantities of vitamin B or vitamin G could be obtained when the respective diets differed only in the kind of carbohydrate. This difference in growth response became quite marked when sucrose-containing diets were compared to dextrin-containing diets. It was believed that by feeding groups of rats a series of diets of constant composition, in which the carbohydrate component was made up of various quantities of sucrose and dextrin, other interesting data would be obtained. In consequence of this fact, a series of six diets was formulated and prepared which ranged in dextrin content from 0 to 71 per cent and in sucrose content from 71 to 0 per cent.

Diet 353 (fig. 7), which contained 71 per cent sucrose and no dextrin, was fed to a group of eight animals. Each animal of the group lost in body weight from the beginning, and by the seventeenth day two of the eight animals were dead. The feeding of the vitamin B concentrate was started at this time, as it was thought that the remaining animals could not survive a 21-day depletion period on the diet and yet retain sufficient vitality for further use in the feeding test. Two more animals died, one on the eighteenth and the other on the twenty-first day. The remaining animals made a definite growth response as the result of receiving the daily allotment of the vitamin B concentrate. The growth-stimulating effect lasted for about 3 weeks when the four surviving animals

began to lose weight gradually for a period of 3 weeks or until the diet was further supplemented by daily additions of autoclaved yeast. During the 2 weeks previous to the addition of the autoclaved yeast, all four of the animals manifested some of the external symptoms usually associated with vitamin G deficiency in the rat. These symptoms include salivation, ulceration of the lining of the mouth and tongue,

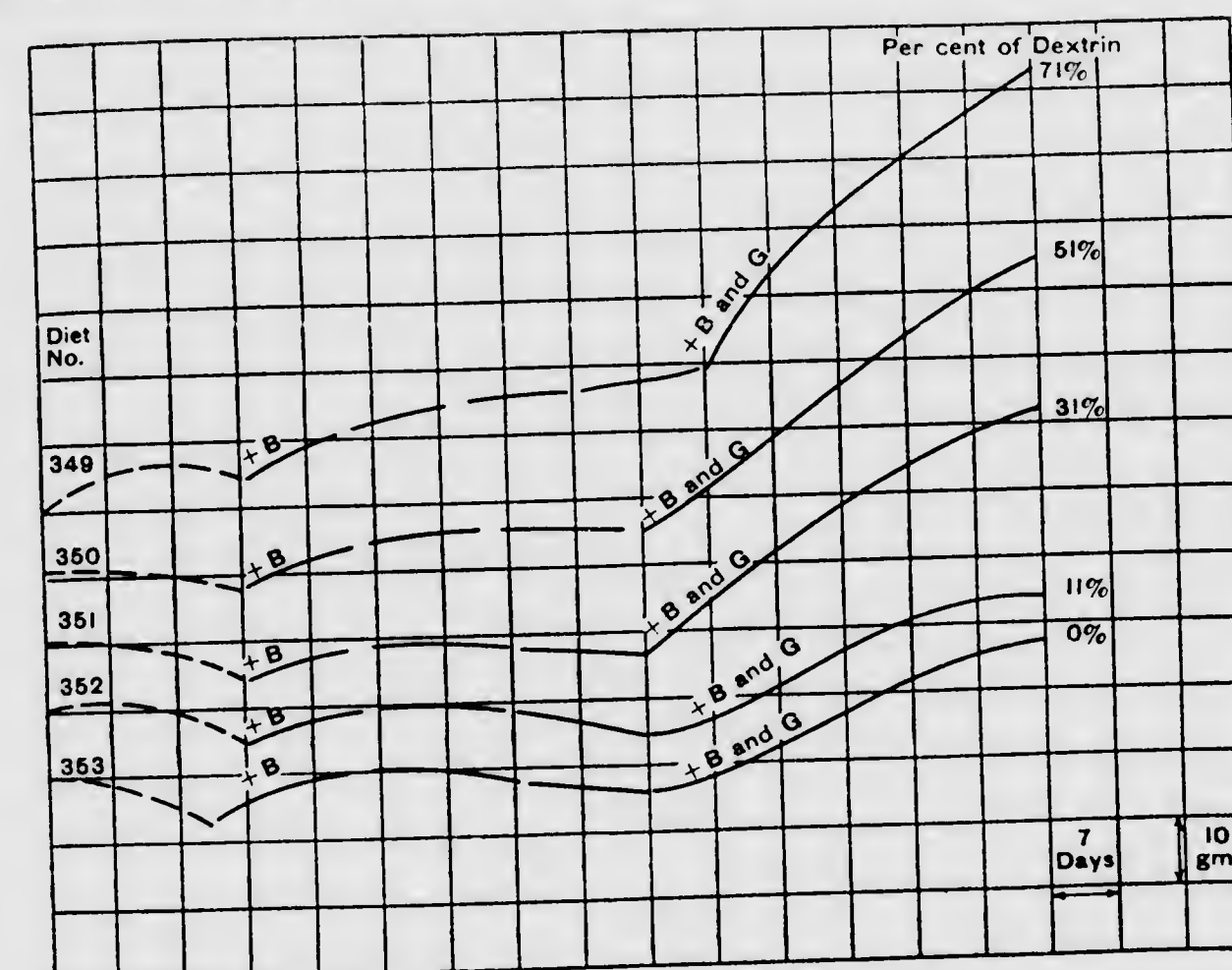


Fig. 7 Showing the average growth responses made by groups of rats receiving, 1) vitamin B deficient diets of varying dextrin content, 2) these diets supplemented by daily allotments of 0.1 ml. of the vitamin B concentrate, and, 3) these diets supplemented with daily allotments of both 0.1 ml. of the vitamin B concentrate and 0.3 gm. of the vitamin G fraction.

inflamed feet and tail, frequently followed by some signs of necrosis, alopecia areata, and distinct diarrhea. When 0.3 gm. of autoclaved yeast was given to each animal daily, there was some delay in the resumption of growth, but the external symptoms showed marked improvement by the end of the first week. From that point, the animals grew at a fairly uniform rate for the remaining 5 weeks and improved in both activity and general appearance.

On decreasing the sucrose content of the diet to 60 per cent and adding 11 per cent of dextrin as in diet 352, the nutritional picture in general was not greatly improved. The most noticeable difference occurred during the preliminary depletion period. When the sucrose content of the diet was further reduced to 40 per cent and the dextrin increased to 31 per cent, as in diet 351, a distinctly improved nutritional picture was obtained. Further increases of dextrin to 51 per cent and to 71 per cent, as in diets 350 and 349, yielded still greater growth responses and corresponding improvement in the appearance of the experimental animals. Experiments are now in progress which we hope will throw some light upon the mechanism by which the type of carbohydrate exerts its growth-promoting or growth-inhibiting effect, while all other known constituents of the diets are identical as to quality and quantity.

Inspection of figures 2 to 7, inclusive, leads to the general conclusion that a definite correlation exists between the growth responses obtained and the composition of the diets fed. This relationship holds quite well within each of the respective series of diets. But when one compares the results obtained by feeding diets 329, 338 and 350 (table 2 and figs. 3, 5 and 7), this correlation does not appear to be quite so pronounced. These three diets were identical in composition and were fed to three different groups of rats under very similar conditions, except the season of the year in which the experiments were carried out. Diets 306 and 319 were also identical in composition to the above diets but each was fed under different conditions and for this reason the results are not comparable to the results obtained on the above diets.

Consideration of the approximate caloric intake of the various groups of rats during the three phases of the experiments shows that the vitamin B concentrate alone was not always effective in reducing the energy required to produce a unit of growth. In fact, no definite relationship appeared to exist between the quantity of ingested energy and the resultant

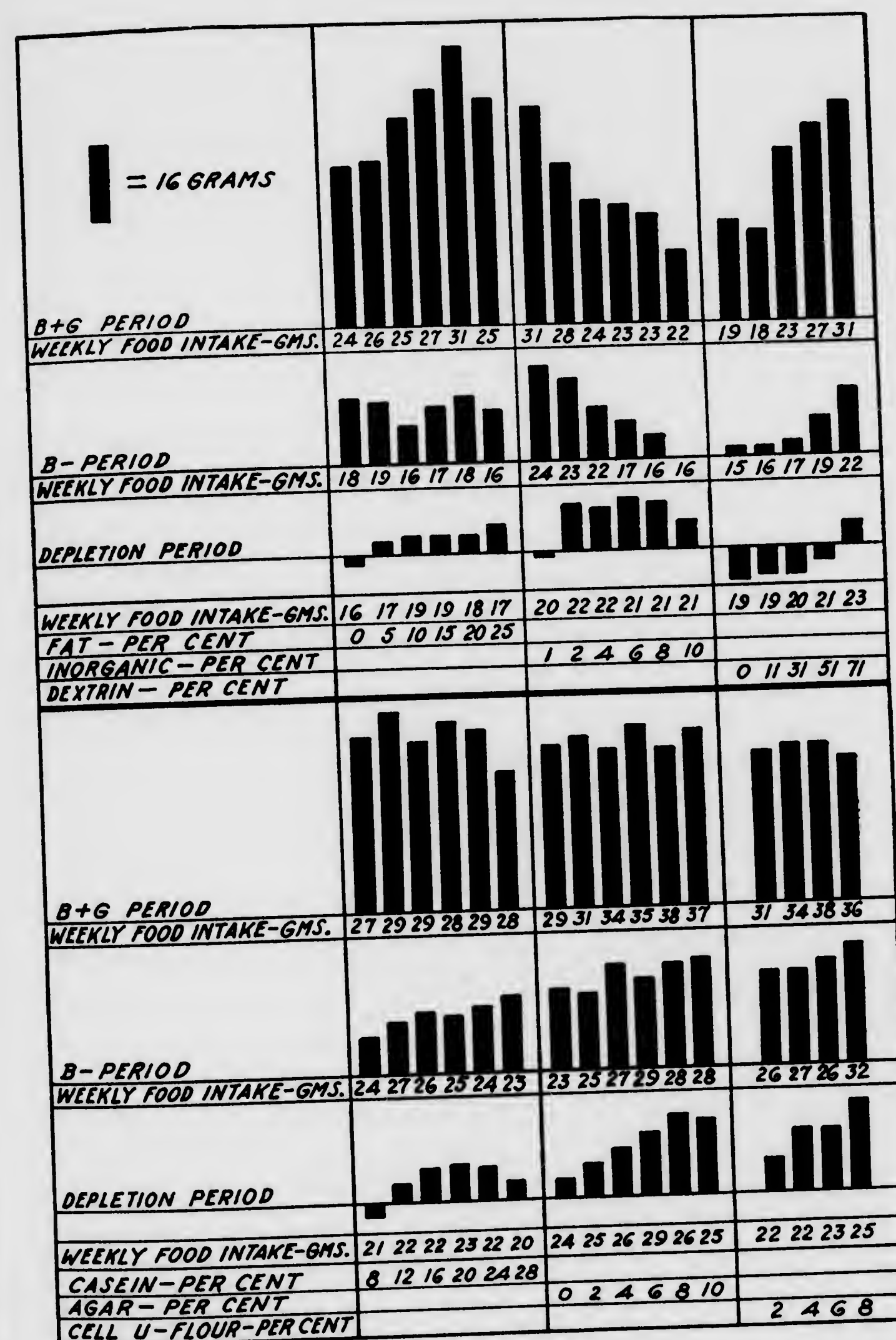


Fig. 8 Showing the relation of the quantity of the various diets consumed to the resultant growth responses made during the three phases of the feeding trials.

rate of growth when groups of young rats were fed the several series of diets, first unsupplemented and then supplemented with a vitamin B concentrate. When these various diets were further supplemented with vitamin G, there was a very noticeable decrease in the quantities of energy required for a unit of growth. The data obtained are insufficient to justify the assumption that vitamin G is unique in its ability to bring about a more efficient utilization of energy for growth. Other essential nutrients would probably be found equally effective in this respect, were they the limiting factors in an otherwise complete diet.

Figure 8 reveals some interesting relationships which were found to exist between the quantity of the various diets consumed and the resultant growth produced. These relationships, as shown among the diets of both the inorganic and the dextrin series, appear to be highly significant.

CONCLUSIONS

1. Rats became depleted of their vitamin B reserve somewhat less readily when the basal diet contained increasing amounts of fat. The addition of increasing amounts of fats appeared to have no effect on the vitamin G requirement of the rat. The most satisfactory growth responses (when rats were receiving a somewhat restricted intake of vitamins B and G) occurred when the fat content ranged from 15 to 20 per cent.

2. The rat's requirement for vitamin G is greater as the mineral salts are increased. And, conversely, the lowering of the percentage of mineral salts in the diet seemed to have a sparing effect on this vitamin.

3. No evidence could be obtained to show that variations in the protein content of the diet have an effect on the utilization of vitamins B and G.

4. Increasing amounts of fiber in the form of agar and CellU flour possessed a definite sparing effect on vitamins B and G utilization. The beneficial effect of fiber is thought to be due to the production of more favorable conditions for the growth of microorganisms in the digestive tract.

5. The demand of the rat for vitamins B and G increases when sucrose is fed as the sole source of carbohydrate, while the need for these vitamins decreases as the sucrose is replaced by dextrin.

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RELATION OF CERTAIN AMINO ACIDS TO CARBON DIOXIDE AND MYCELIUM PRODUCTION OF *FUSARIUM* *OXYSPORUM*

ARTHUR K. ANDERSON AND KATHRYN EMMART

(WITH ONE FIGURE)

Introduction

It has been known for many years that the ingestion of foods, especially proteins, stimulates metabolism in animals. LUSK (11, 12) has shown that certain amino acids produce the same stimulation as proteins. He believes that the stimulatory effect of proteins can be accounted for by the active amino acids which they contain. LUSK (10) has shown that glycine, alanine, leucine, and tyrosine stimulate oxidation in the order given, while glutamic acid does not. ATKINSON and LUSK (3) added aspartic acid and asparagin to the list of those which showed no effect. RAPPORT and BEARD (15) found phenylalanine to have the greatest stimulatory effect of any amino acid. ORT and BOLLMAN (14) found that cystine, glycine, alanine, phenylalanine, leucine, histidine, and valine catalyzed the action of hydrogen peroxide on dextrose, while glutamic acid, aspartic acid, and tyrosine had no effect.

With regard to the effect of amino acids on plant metabolism there is a considerable literature. In green plants SPOHR and MCGEE (17) noted that the leaves of *Helianthus annuus* when fed a glycine solution consumed glucose more rapidly, and showed a greater respiratory activity as measured by carbon dioxide evolution, than leaves not so fed. KOSER (9) observed that certain amino acids supported growth of some bacteria but not others. GORDON and MCLEOD (6) noticed no marked effect of amino acids on hardy bacterial organisms such as *B. coli*, but that they stimulated the growth of certain delicate organisms. Several workers (8, 13, 20) have shown that certain amino acids influence the amount and size of yeast cells and also yeast activity in fermentation.

With regard to the effect of amino acids on fungi, SCHULZE (16) has shown that leucine and tryosine serve as a food material for *Penicillium glaucum*. TRIS (18) found that leucine, glutamic acid, histidine, cystine, and glucosamine stimulated the germination of spores of *Phycomyces nitens*. HERZOG and SALADIN (7) have reported a decided stimulation in the metabolism of *Penicillium glaucum*, as measured by carbon dioxide production, after the addition of leucine to the medium.

In this laboratory considerable work has been done on the metabolism of *Fusarium oxysporum*. ANDERSON, EVERITT, and ADAMS (2) have shown that the main products of metabolism of this organism, when grown in a medium in which glucose is the only source of carbon, are carbon dioxide and ethyl alcohol. Since *Fusarium oxysporum* grows well in a medium containing no organic nitrogen, it was felt that this organism offered an excellent opportunity to study the stimulatory effect of amino acids on fungus metabolism. This paper reports a study of the rate of metabolism of *F. oxysporum* in glucose media containing glycine, leucine, tyrosine, and aspartic acid as measured by carbon dioxide production. Glycine, leucine, and tyrosine were selected as amino acids which might be expected to produce a stimulation in metabolism and the aspartic acid as one which might not.

Methods

The culture of *Fusarium oxysporum* used was obtained from the Division of Plant Pathology at the University of Minnesota. The stock medium was that used by ANDERSON (1) in his work on *F. lini* and had the following composition:

Ammonium nitrate	1.00 gm.
Monopotassium phosphate	0.50 "
Magnesium sulphate	0.25 "
Glucose	20.00 "
Water to make	1000 cc.

Using this as a basis, the amino acid media were prepared by adding 0.5 gm. carbon equivalent of an acid for each 300 cc. of stock medium (the amount used in each culture flask), i.e., 1.5635 gm. of glycine, 0.9105 gm. of leucine, 0.8385 gm. of tyrosine, and 1.386 gm. of aspartic acid. Two controls were run, control A being the stock solution alone and control B being the same as control A except that 0.5 gm. of carbon in the form of glucose (1.25 gm.) was added to each flask. Three hundred cc. of each medium were placed in 500-cc. pyrex Erlenmeyer flasks. Triplicate experiments were run in each case. Each flask was closed by a two-hole rubber stopper through which extended two glass tubes. Cotton plugs were placed in the outer end of each tube to prevent contamination.

After autoclaving at 15 pounds' pressure for 20 minutes and cooling, each flask was inoculated with 10 cc. of spore and mycelium suspension. The stoppers were sealed with paraffin and the ends of the glass tubes were closed with rubber tubes and screw clamps.

Separate samples of all solutions were autoclaved with the culture flasks and, after cooling, their pH values were determined by the electrometric

method using a quinhydrone electrode. During the experiment the flasks were kept on laboratory desks at room temperature.

Carbon dioxide determinations were made at frequent intervals by aeration into TRUOG (19) towers containing barium hydroxide solution. The excess barium hydroxide was then titrated with 0.1 N hydrochloric acid using phenolphthalein as an indicator.

The cultures were allowed to grow for 229 days, at which time two sets, those containing glycine and aspartic acid, were opened as it was planned to terminate the experiment at that time. However, the others were then allowed to grow longer, 270 days in all. After opening, the cultures were immediately filtered through Gooch crucibles and the mycelia washed, the washings being discarded. The mycelia were dried to constant weight at 100° C. The filtrates were used for the determination of pH and of residual glucose by the method of FOLIN and WU (5).

Presentation of data

Figure 1 and table I present the results of this investigation. Figure 1 shows the total amount of carbon dioxide produced during growth. This amount is the average of triplicate determinations for each culture medium.

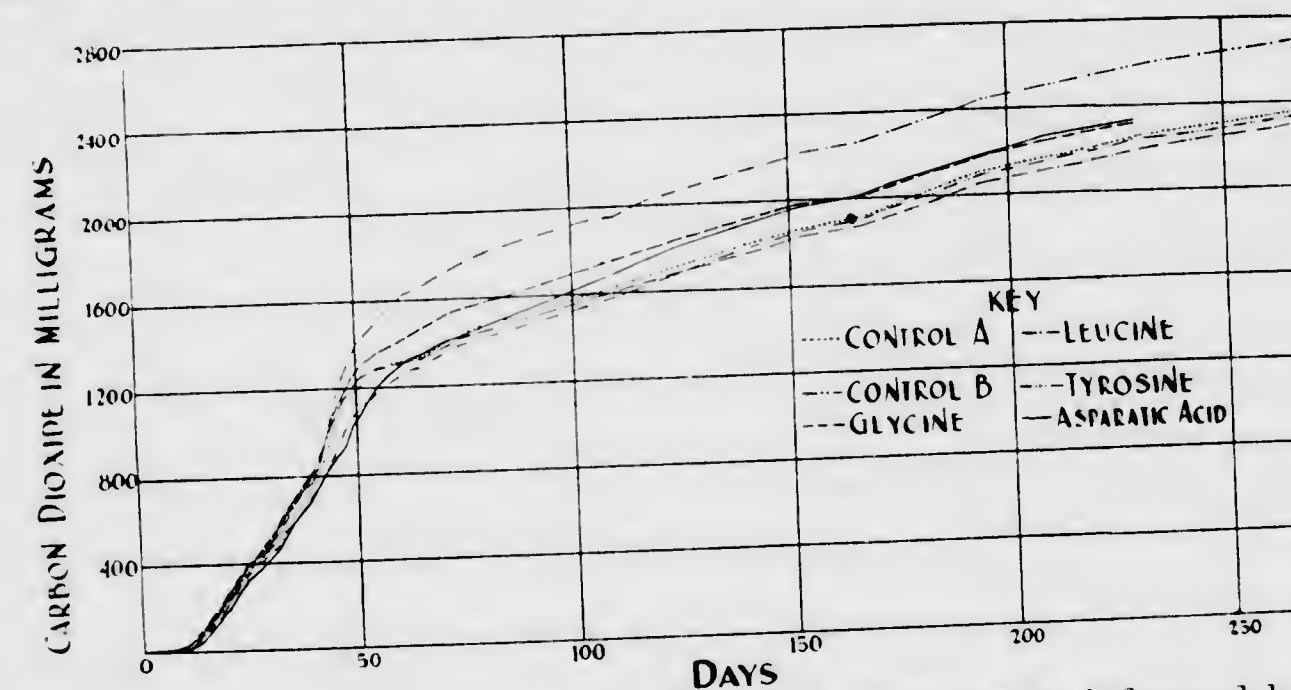


FIG. 1. Carbon dioxide production by *Fusarium oxysporum* as influenced by certain amino acids.

After a determination, the weight obtained from each culture was added to that previously obtained, so that the amount given for any day represents the total carbon dioxide produced from the time of inoculation to that day. It can be observed that control B, the medium modified by the addition of glucose, produces the greatest amount of carbon dioxide, which indicates that the carbon in glucose is the best source for carbon dioxide production by the fungus. The curve for the carbon dioxide produced by the fungus on the medium modified by aspartic acid is below all the others until the

53rd day, after which it gradually rises until on the 229th day it is above all except that for control B. The high initial acidity of this medium evidently causes the early retardation of metabolism, and, as the pH rises, a more optimum condition is reached, thereby producing a greater activity.

It can be observed that the glycine, as well as the aspartic acid medium, gave more carbon dioxide than control A. These two amino acids are therefore used as a carbon source by *Fusarium oxysporum*, although not to the same extent as an equal carbon equivalent of glucose. Tyrosine slows the rate of carbon dioxide production, while leucine has the greatest retarding effect. The comparisons are best made at 229 days, but by observing the chart one can see that the same ratio would exist if the curves for glycine and aspartic acid had been continued.

Table I gives a résumé of this investigation. The figures given represent the averages of triplicate determinations. It is unfortunate that the period of growth of the cultures containing glycine and aspartic acid was not so long as that of the others. It is therefore difficult to make comparisons. One can observe that the weights of mycelia obtained from the two cultures are even greater than that from control B in which growth was continued over a longer period of time. This might be accounted for by the beginning of autolysis when the fungus is allowed to grow after most of the glucose is consumed. On the other hand, since tyrosine produces a slightly greater weight than control B and leucine only a slightly smaller one, it appears that the amino acids studied, with the exception of leucine, are utilized to a greater extent than an equal carbon equivalent of glucose to produce fungus body.

The small amounts of residual glucose noted in the table show that most of the 6 gm. of glucose originally present in each flask is utilized during growth. The high values obtained for the cultures containing glycine and tyrosine are not significant because these two amino acids were found to have a slight reducing action on the reagents used in the glucose determination.

In every culture the hydrogen-ion concentration decreases during growth. The lowest original pH is for the media containing aspartic acid. This is due to the fact that this amino acid is a dicarboxylic acid. For the other media, the range of pH is from 4.6 for control B to 5.3 for that containing tyrosine. The final pH of all the media is 7.5 or above. Comparisons are again rather difficult on account of the difference in length of the period of growth of the cultures. The greatest increase in pH is noted for the medium containing aspartic acid, a change from 3.1 to 8.0. The medium containing glycine had a final pH of 8.1. As previously stated, these two amino acids, although the fungus was allowed to grow on them for a shorter length of time, produce the greatest weight of mycelium, and

TABLE I
TOTAL CARBON DIOXIDE AND MYCELIUM PRODUCTION, RESIDUAL GLUCOSE, AND ORIGINAL AND FINAL pH OF THE MEDIA

DESCRIPTION OF MEDIA	PERIOD OF GROWTH	WEIGHT		pH	
		CARBON DIOXIDE	DRY MATTER IN MYCELIUM	RESIDUAL GLUCOSE	ORIGINAL FINAL
		mg.	mg.	mg.	
Controls	A: stock medium alone	2366.0	376.3	12.7	4.7 7.7
	B: stock medium plus glucose	2713.6	406.3	20.6	4.6 7.6
Media modified by addition of amino acids	Glycine	2322.4	483.8	37.6	4.8 8.1
	l-leucine	2321.9	403.1	28.8	4.9 7.7
	l-tyrosine	2351.6	410.9	67.1	5.3 7.5
	l-aspartic acid	2341.0	563.0	20.0	3.1 8.0

up to the time of the opening of the cultures, the greatest amount of carbon dioxide with the exception of control B.

Conclusions

At the outset of this experiment it was expected that certain amino acids which are known to produce a stimulation of metabolism in animals might do so with the fungus, *Fusarium oxysporum*. It is apparent from figure 1 that there is no such stimulatory effect as measured by carbon dioxide production. Cultures containing glucose in amounts equivalent in carbon content to the carbon of the added amino acids show just as rapid a production of carbon dioxide as those containing amino acids. In the end cultures containing added glucose produce much more carbon dioxide than do any of the cultures containing amino acids. Aspartic acid and glycine are utilized by the fungus for the production of carbon dioxide, while leucine and tyrosine retard the production of carbon dioxide.

All of the amino acids studied are utilized by the fungus in the production of mycelium. With the exception of leucine they are all a better source of mycelium building material than glucose. Aspartic acid is outstanding in its ability to produce mycelium.

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Reprinted from SCIENCE, December 21, 1934, Vol. 80,
No. 2086, page 587.

MISCONCEPTIONS RELATIVE TO THE MINERAL COMPOSITION OF PLANTS

THE recent papers¹ on a comparison of the percentage composition of the dandelion with certain other edible plants are indicative of the prevalence of the idea that the mineral composition of each species is a fixed characteristic of that species. This concept is a legacy from the teachings of Liebig known as Liebig's "law of the minimum,"² from which the deduction was made that the principal mineral nutrient elements are absorbed only in certain definite and fixed proportions characteristic of the species.

The influence of this concept resulted in the neglect by the majority of those investigators, who, as a means of diagnosis, made analyses of an entire plant (or plants) periodically during the vegetative season or at maturity, to support the analyses by reference to the conditions—soil, cultural and meteorological—under which the plants were grown; and who were thereby led to conclude that the results obtained were established *ad universum et ad aeternum*, whereas, in fact, analyses had been made only of a particular plant (or plants) grown in a particular soil in a particular year. In this way the extreme sensitivity, the flexibility (plasticity) and "le mode d'alimentation" of a plant, readily recognized by the method of foliar diagnosis,³ were not clearly discerned.

The fact that has been hitherto and is still at present lost sight of is that comparison of the quantitative

¹ E. Bennett, SCIENCE, 80: 142, 1934; G. E. Youngburg, *ibid.*, 80: 337, 1934.

² Walter Thomas, SCIENCE, 70: 382-384, 1929.

³ Walter Thomas, *Soil Science*, 33: 1-20, 1932; and the references to the papers by H. Lagatu and L. Maume cited therein.

relations between the elements during the growth of a particular species subjected to different growth conditions—cultural and meteorological—can not be made without a key of interpretation and that consequently comparisons between the composition of different species with respect to any of the dominant or accessory mineral elements also can not be made without a key of interpretation. The problem for the future is to work this out.

WALTER THOMAS

THE PENNSYLVANIA STATE COLLEGE

A MANUAL OF BIOCHEMISTRY. *J. F. McClendon*, Professor of Physiological Chemistry, University of Minnesota Medical School. First edition. John Wiley & Sons, Inc., New York City, 1934. vii + 381 pp. 58 Figs. 15 × 23 cm. \$5.00.

This book is an outgrowth of mimeographed material which has apparently been used in a course in physiological chemistry at the University of Minnesota for some time. The order followed in this book is a distinct departure from the conventional arrangement found in recognized texts on physiological chemistry. The greater part of the book concerns itself with elements and compounds of biological importance, with a discussion involving the function and importance of each.

The book is divided into six parts. Part I deals with physical chemistry as related to biology and includes such subjects as colloids, catalysis, calorimetry, internal secretions, and ionic equilibria. Part II, which occupies 42 pages, deals with inorganic elements and compounds. In Part III the organic compounds of biological interest are discussed. Part IV is a brief summary of foods, digestion, metabolism, and excretion. Part V is devoted to laboratory directions involving the quantitative determination of important organic and inorganic constituents of biological materials. Part VI is a table of 1,000 elements and compounds of importance in biology, giving their molecular weight, melting point, boiling point, density, and solubility in water, alcohol and ether. In most cases a statement is included giving their occurrence, use, or importance in biology.

One of the features of the book which should prove of value to the student is the citation of numerous references at the conclusion of each subject discussed.

One of the things that impresses the reader is the inclusion of much material which is not found in other books on physiological chemistry. This is especially noticeable in the section on inorganic elements. The section on iodine and its relation to goiter is especially good. Following the section on the radio-active elements, considerable space is devoted to radiation, which includes discussions of such subjects as radio and cosmic rays. The subject of diabetes and insulin is well discussed and many ideas are presented which are not found in other books.

The section on laboratory work is quite different from other laboratory guides in physiological chemistry. All the experiments are quantitative in nature, and micro technic is emphasized. Methods are given for the determination of many substances for which methods are not available in other books. Many of the methods are new.

Perhaps the most important criticism that can be made of the book is the brevity with which many subjects are dispensed with. In many cases simply the name and the chemical formula of a compound are given. Frequently the discussion of a compound is indefinite and fragmentary and leaves one with the feeling that an enlargement of the discussion would make the information of more value and interest to the student. For example, on page 277 the following compounds are dispensed with as follows: "Nicotine paralyzes certain nerve ganglia when a highly concentrated solution is applied to them locally." "Cinchophen, atophan, is related to quinine. It has been given to patients with gout." "Quinine is used to cure malaria. Its use was discovered by South American Indians."

On page 29, specific dynamic action of protein is discussed. One is left with the impression that proteins alone have a specific dynamic effect. Mention should be made of the fact that carbohydrates and fats produce a similar effect. On page 287 the following statement is made: "The digestion of meat in the stomach produces some substance which *cures* pernicious anemia." The writer feels that some other term should be substituted for the word "cures."

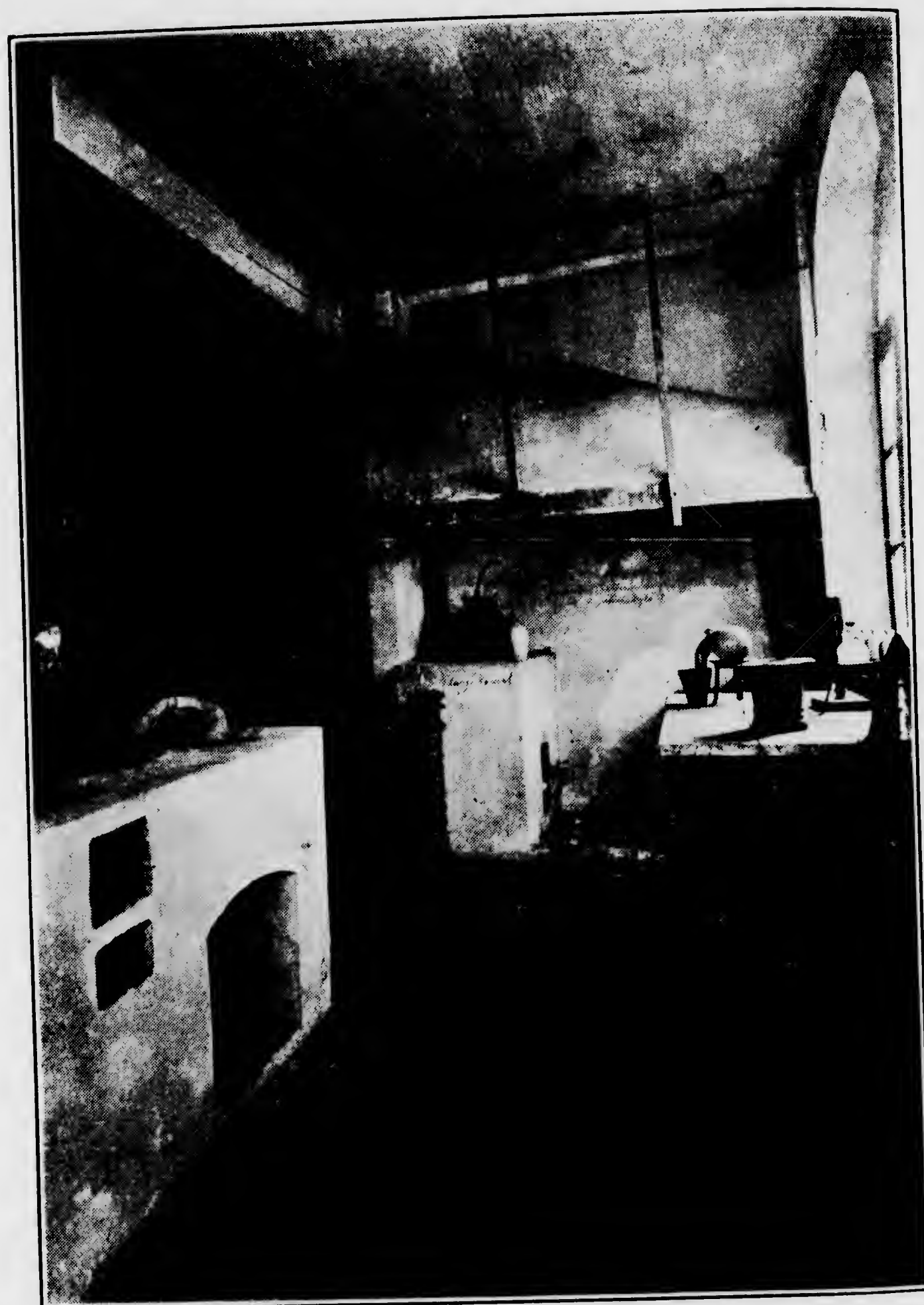
To one who is accustomed to teaching physiological chemistry in the conventional manner it is difficult to see how this book could replace the excellent texts which are now available. For a more general course in biochemistry it will be useful. As a reference book it is excellent and should find a place in the library of everyone interested in the field of biochemistry.

A. K. ANDERSON

SOME IMPRESSIONS OF BIOCHEMICAL RESEARCH
WORK IN GERMANY

R. ADAMS DUTCHER

Reprinted from The Hexagon of Alpha Chi Sigma, February, 1935



Old Laboratory (Liebig's) in Giessen

The Hexagon of Alpha Chi Sigma

February
1935



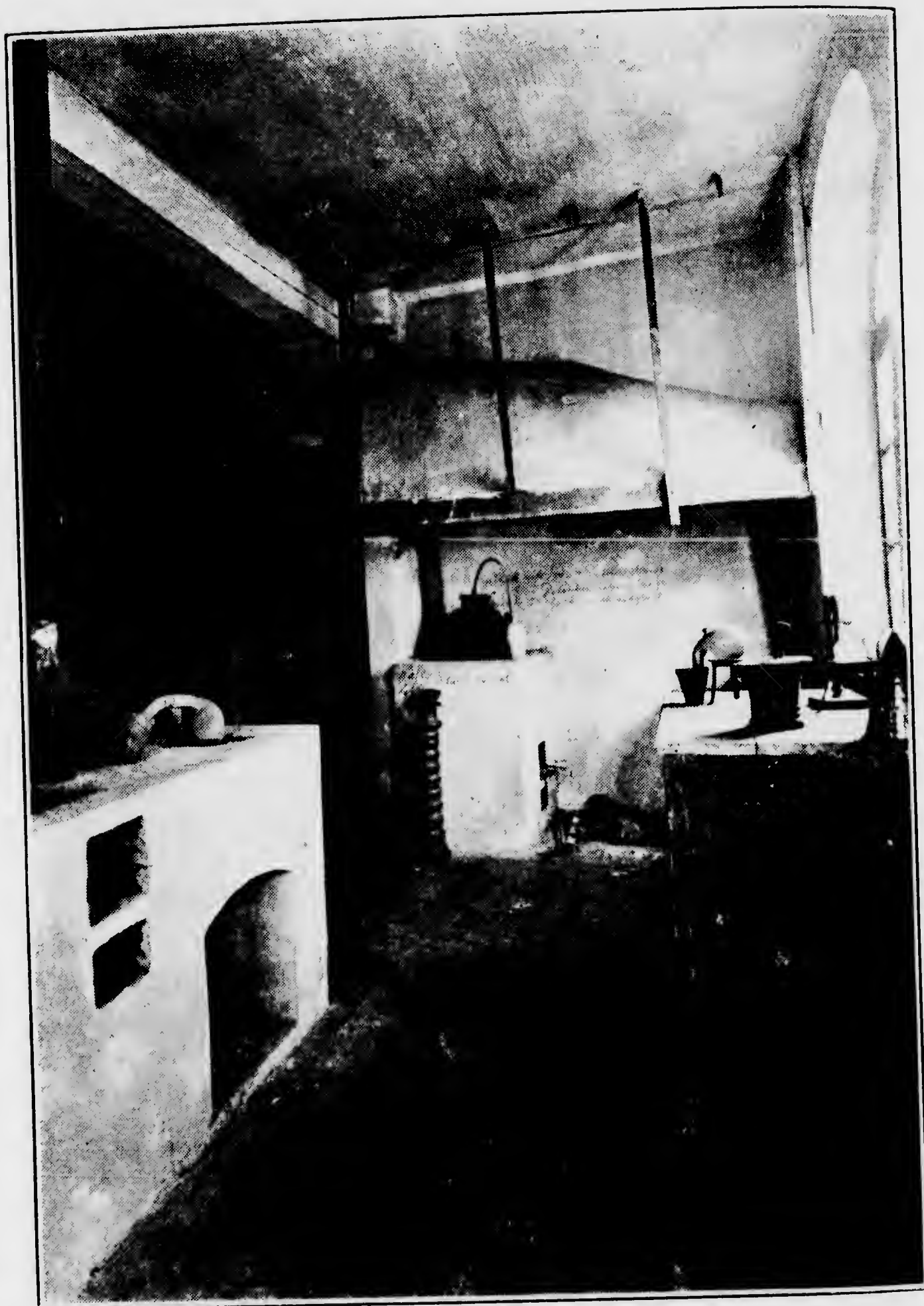
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SOME IMPRESSIONS OF BIOCHEMICAL RESEARCH WORK IN GERMANY

By R. Adams Dutcher, *Delta*

EDITOR'S NOTE.—Members of the fraternity find their way into all parts of the world and from time to time their experiences or reactions have appeared in the HEXAGON. It is with pleasure that we introduce Professor R. Adams Dutcher and his "Impressions of Biochemical Research Work in Germany." During the spring and summer of 1934, Professor Dutcher traveled in Europe as an Oberlaender Trust Fellow of the Carl Schurz Foundation and also as collaborator of the Bureau of Chemistry and Soils of the U. S. Department of Agriculture. Five months were spent in Germany with side trips to France, Austria, Switzerland, and Holland, and brief visits to Sweden, Norway, and England. During his stay in Europe he interviewed many scientists and government officials with reference to various phases of chemistry and biochemistry relating to agricultural and public health problems. . . . Professor Dutcher received his undergraduate work at the South Dakota State College, and carried on graduate work at the same institution, the University of Missouri, University of Minnesota, and University of Illinois. He has held teaching and research positions at South Dakota State College, University of Missouri, University of Illinois, Oregon Agricultural College, and University of Minnesota. He installed the first vitamin research laboratory at the University of Minnesota and came to Pennsylvania State College in 1921 as head of the Department of Agricultural and Biological Chemistry, where he is now located. He was initiated by Delta chapter (University of Missouri) October 31, 1910.

WHEN a visitor enters the outer office of a German National Socialist official he is impressed with the number of slogans and mottoes which adorn the walls. One of these, which invariably is placed in the most conspicuous place, reads as follows: "Man kommt nicht in einige Sache dorthin, wo ein neuer Staat gebaut wird." Freely translated, this means "One does not come here for the solution of his own problems when a new government is being created." In my opinion, this slogan expresses the spirit of modern Germany.



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In spite of the above-mentioned admonition, I found that busy officials, industrial executives, teachers, and research workers were willing and ready to drop their work and give me any reasonable amount of their time to show and explain any phase of their work which appealed to my interest as an agricultural biochemist interested in problems relating to nutrition, public health and agriculture. Naturally I endeavored to visit most of the universities and agricultural high schools where biochemical work was being done, but I also had the good fortune to visit a number of industrial laboratories, medical clinics, and government bureaus.

From what I had read prior to embarking for Germany, I had expected to find the country restless and in turmoil. Aside from the continuous marching and singing of the Hitler youth and the S. A. men in brown uniforms, I found the country to be remarkably orderly and quiet. One thing that strikes the foreign traveler in Germany is the fact that there are no street loafers, and during the entire five months' period in which I traveled in Germany I saw no beggars or evidence of poverty. The government has many devices to keep its people fairly busy at various types of work regardless of the fact that they receive very small compensations.

I found the universities actively engaged in teaching and research in spite of the fact that student registration has been curtailed by government order. In the future no young man or woman will be allowed to enter the university until he or she can submit evidence to show that he or she has contributed six months of work on assigned state projects. Toward this end, Germany has established about thirteen hundred "arbeits dienst" or work service camps. Each of these camps has a capacity of about 200 to 225 men. Boys and young men ranging from seventeen years of age to twenty-five must spend at least six months in these camps before being allowed to enter the university or to obtain employment in industry or with the government. As a result, this project alone keeps about 250,000 young men busy at useful work which occupies their time and thought from five o'clock in the morning until ten o'clock at night. This is undoubtedly one of the reasons why the traveler in Germany sees so few loafers and unemployed people on the streets. Space will not permit a description of other government projects which accomplish a similar purpose. I had the pleasure of visiting two of these *arbeits dienst* camps and had an opportunity to learn at first hand how these young men are being trained in the new national socialist philosophy. These camps are scattered throughout Germany and much valuable work is being done at a very low cost in the reclaiming of waste lands, conservation of forests, repair of rural highways, construction of community housing projects, and similar governmental activities.

The new order has affected university work in a number of ways,

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some institutes or departments being more active than usual while other institutes seem to be handicapped by a lack of finances and personnel. A short time prior to my arrival in Germany (March 9, 1934) the government had just passed its anti-vivisection law. This law aims to eliminate unnecessary vivisection work in medical schools and biological laboratories by requiring permits from responsible heads who are held responsible to the State for any work that might be done. For the first month or two it was quite evident that the new law had a detrimental effect on many types of research work owing to the fact that responsible heads of institutes were unwilling to allow experimental work to continue until the permits were obtained. Before three months had elapsed permits had been issued to all important laboratories with the result that important work continued as usual.

Rectors, who hold positions similar to those held by college presidents in this country, are still chosen for two-year periods by the members of the university faculty; but the new law requires that the faculty nominate three men in order of preference. These names are submitted to the Educational Ministry which puts its stamp of approval on one of the three nominees. I obtained the impression that the faculties, or university senates, nominate these men with great care, with the result that choice number one invariably meets with the approval of the national socialist officials.

Popular and capable professors who can speak with authority in their fields seem to have large classes in spite of the fact that some of them do not meet with the complete approval of the national socialist régime. This is a matter of great importance to the professor



Arbeitsdienst (Work Service) Camp at Bernau

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or teacher in Germany, owing to the fact that his annual income depends to a great extent on the size of his classes, since he obtains a large amount of the fees paid by the students.

Whenever it was possible to do so, I attended lectures in many of the universities and high schools and was impressed with the very high order of the lecture demonstrations conducted in inorganic and organic chemistry. When German students approve of a professor or his lecture or demonstration they show approval by stamping their feet. When the German student wishes to show that he does not approve he shuffles his feet on the floor.

While in Heidelberg, I was interested to learn that returning alumni who had attended the university in the "good old days" were complaining that the "college spirit is rapidly disappearing." They complained that the first-year students were attending classes faithfully to the complete neglect of fraternity life, beer, and song-fests and duels. They complained bitterly that dueling technique, in particular, had deteriorated.

In many cases I obtained the impression that the quality of teaching and research has been seriously affected through the loss of many professors and directors of institutes who were known internationally for the excellence of their work. Many, if not most, of these were Jews who lost their positions as the result of the unfortunate methods adopted by the Hitler régime. Some of these men had left the country, others had remained in Germany and were living on their savings, while others had been pensioned by the government and were living in their university towns, quietly but socially apart. Some Jewish professors of international reputation still retain their university positions, but in nearly every case it was my observation that their institutes were supported wholly, or in part, by funds from the Rockefeller Foundation.

Some institutes had excellent physical plants which were wonderfully equipped. As a rule these institutes not only received money from the local German states but they also had funds from the Notgemeinschaft (government funds) with perhaps some assistance from the Rockefeller Foundation or similar organizations. Other institutes with directors of prominence might be a part of the same university and yet have budgets so small that it was impossible to do really worthwhile research.

In most of the universities and high schools I found that the research programs were being conducted along the same lines as in previous years. In other words, I was unable to find that the new government had had any great influence on the research program. Some laboratories, particularly those in agricultural high schools, were conducting new projects at the request of their local states or at the request of the government. All of these projects, naturally,

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were pointed toward making Germany self-sustaining as soon as possible. Such projects were along the line of soil and crop improvement, improvement of food equality and quantity, a search for cheaper sources of fertilizer materials, the utilization of cheap by-products, the improvement of bread wheat and other cereals, the production of oil-bearing seeds, the utilization of peat as a substitute for farm manure in building up the organic composition of sandy soils, and many other similar projects which have to do with increased food production. I was informed by the agricultural ministry that Germany was already over-produced on pork, wheat, and rye. Some very excellent research work is being done in the agricultural experiment stations and agricultural high schools of Germany but most of this work is along soil and crop improvement lines. It is my impression that we surpass Germany in many fields of agricultural research and that they can learn much from us today regarding the practical applications of scientific methods. They are just awakening to the importance of more research work along dairy and horticultural lines, and particularly in the field of plant pathology. While they are far in advance of us in the application of theoretical organic chemistry to many agricultural problems, we are equally far advanced in the application of scientific principles to agricultural practice. This is true in the field of human and animal nutrition, and particularly in the field of vitamin therapy. They are just beginning to appreciate the value of more milk in the diet of the growing child, and I was interested to see that it required considerable propaganda on the part of the government to make the people appreciate the fact that milk is a



Panorama of Heidelberg looking toward the Neckar River from the Grounds of the Old Castle.

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valuable and necessary article of the daily diet. I saw a government propaganda film at the Wintergarten, Berlin, which not only showed the public that milk was a good food for babies and growing children but, at the end of the film, a big, burly policeman purchased and drank a glass of milk to show the people that it was no longer a baby food. Dr. Mangold, eminent nutrition worker in Berlin, informed me that he had had the unusual experience not many days before, of seeing a driver of a beer wagon drive up to a dairy store and purchase a glass of milk.

I had the privilege of visiting the Kali Syndikat, Germany's large potash exporting syndicate, and I found that they are conducting an enormous number of agricultural projects of a fundamental and practical nature. Naturally all of their experimental work is along production lines with the view of producing plants of better quality and quantity. They informed me that most of their experimental work at the present time is confined to "improved quality" studies. I also had the delightful experience, as their guest, to visit a typical German potash mine.

Among the other industrial laboratories which I visited in Germany were the Schering-Kalbaum plant in Berlin, and the Merck plant in Darmstadt. These manufacturing plants are devoted to the manufacture of biological products and chemical and pharmaceutical products. The research work being conducted in the laboratories of these industrial organizations is of a very high order. I was interested particularly in the work on the sex hormones by Dr. Schoeller and his staff at the Schering-Kalbaum laboratories in Berlin. They have isolated the male and female sex hormones from urine and these have been obtained in crystalline form and their constitution or configuration have been determined. The male hormone has the empirical formula $C_{19}H_{30}O_2$ while the female hormone has the formula $C_{18}H_{22}O_2$. Dr. Schoeller informed me that their structure is quite similar, both of them being related to 1-Keto, 1-2-3-4 tetrahydrophenanthrene. He also informed me that the male hormone could be increased in potency about five times by submitting it to hydrogenation under pressure.

I also had the pleasure and privilege of having the Aschheim pregnancy test demonstrated for me by Professor Aschheim himself. This test is carried out on ovariectomized mice, which are injected with the suspected urine. Pregnant urines cause estrus and certain very definite physiological changes. Dr. Aschheim also gave me the interesting information that he had extracted materials from peat, brown coal, and petroleum which had definite estrus-producing effects.

It is impossible to describe all the interesting experimental work that I saw, nor is it possible even to mention the more important work in all of the universities and high schools that I visited. I am therefore taking the liberty to pick out a few of these institutions in order

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to give the reader some idea of the types of research programs which are being conducted in typical German universities at the present time.

I found that Professor Emil Abderhalden, at the University of Halle, was conducting a very active research program along lines which have made this laboratory famous in the past. Dr. Abderhalden and his students are continuing their work on the chemistry of proteins, the synthesis of peptides, and on the isolation and identification of specific proteolytic enzymes and their action. He is carrying on some very significant work on the interrelationship between hormones and vitamins and the influence of vitamin B on food consumption and metabolism.

I had the interesting experience in Munich of interviewing three famous Nobel prize winners; namely, Professors Hans Fischer, Heinrich Wieland, and Richard Willstätter. Professor Fischer is continuing his research work on the plant and blood pigments, and he had a large number of students and assistants working on pigment research, hemin, synthetic pyrroles, and similar chemical substances.

The Third Reich has had no apparent effect on the research program of Professor Wieland, for I found that he and his assistants were still working on oxidation changes in living cells, isolation of metabolic by-products, and the intermediate steps in various oxidation reactions and other problems relating to his theory of cell respiration. He was also doing work on the respiratory enzymes, the synthesis of biological compounds of various types as well as the isolation and identification of the toad poisons.

I was interested particularly in this institute for it was here that Justus Von Liebig terminated his active career as Director of the Institute. He was succeeded by Von Baeyer, and Von Baeyer was succeeded by Willstätter. Professor Willstätter retired about nine years ago and was succeeded by Professor Wieland. Wieland has set aside and dedicated a laboratory in the institute which is known as the Willstätter Laboratory, and although Professor Willstätter has not entered this laboratory since he retired nine years ago, his students still have the privilege of working on his problems in the institute. I had the delightful experience of having tea at Professor Willstätter's home and I found that he is not only an authority on chemistry, but he is well known as a grower of roses. He showed me with particular pride a beautiful rose which has been developed in recent years and is known as the Richard Willstätter Rose.

I found that Dr. Carl Freudenberg, Director of the Institute of Chemistry at the University of Heidelberg, had a very interesting and varied research program. This institute had once been headed by Bunsen, whose old laboratory is still preserved just as it was when he worked in Heidelberg. Bunsen was succeeded by Victor Meyer,

and I had the pleasure of listening to a lecture on organic chemistry by Professor Ziegler in the old Victor Meyer lecture room. Victor Meyer was succeeded by Curtius, and Curtius in turn by Freudenberg. The names of former students and teachers at Heidelberg read like a list of Nobel prize winners as you find such names as Bodenstein, Kekule, Von Baeyer, Beilstein, Landholt, Crum-Browne, Erlemeyer, Treadwell, Gatterman, etc. Dr. Freudenberg pointed with considerable pride to the desk where Professor Moses Gomberg, of Ann Arbor, Michigan, had done his work as a student.

Freudenberg and his research assistants are working on such problems as insulin function and structure, the formation of crystalline dextrin by special strains of bacteria, decomposition products and derivatives of pituitary hormones, properties, configuration, and derivatives of the sugars, starches, celluloses, and lignins. They have also synthesized a crystalline tri-saccharide and cello-biose. Many other types of research were also in progress.

At the Kaiser Wilhelm Institute for Medical Research at the new medical center in Heidelberg, I interviewed Dr. Richard Kuhn. He and his associates are doing some very excellent work on the chemistry of the carotenoid pigments, and the flavines. The latter problem was of particular interest to me since it has to do with vitamin G (B_2). Professor Kuhn has isolated lactoflavine from milk, hepo-flavine from liver, and ovo-flavine from eggs. These flavines have been crystallized and their chemical formulae have been partially worked out. He informed me that it was necessary to use about 17,000 eggs in order to obtain 50 milligrams of ovo-flavine which has the empirical formula ($C_{17}H_{20}N_4O_6$). When flavine is fed to vitamin-G-deficient rats, Dr. Kuhn obtains a physiological response which seems to be identical, so far as growth is concerned, with the response that is obtained when vitamin G (B_2) is fed. He has not been able to produce the typical dermatitis which is characteristic of vitamin G deficiency, and I was informed by Dr. Harriette Chick in London that she had been unable to prevent or cure the dermatitis of vitamin G deficiency with the flavine. It would appear, therefore, that the flavines may be only a part of the vitamin G molecule, or it may be that vitamin G consists of two or more vitamins.

Dr. Kuhn has shown that the flavines are very susceptible to light, decomposing into very definite chemical substances which no longer possess vitamin G activity. He has also isolated flavine from Dr. Warburg's yellow enzyme. Dr. Kuhn is of the belief that this work opens up an opportunity for speculation in many directions regarding the part that vitamins may play in cell respiration, in enzyme systems, etc.

While I was impressed with the high order of Dr. Kuhn's work so far as the organic chemistry is concerned, I was not particularly im-

pressed by his biological technique for the measurement of vitamin activity. I came away from Germany with the feeling that Dr. A. Scheunert, in the Institute of Animal Physiology at Leipzig, was Germany's best technician so far as the accuracy of (biological) vitamin assays is concerned.

I was particularly interested in the work of Professor A. Windaus and his associates at the University of Göttingen. His institute was equipped with every type of apparatus necessary for chemical research. He was working on the chemistry of Calciferol and vitamin D preparations made from cod liver oil, and he informed me that he had at last determined the configuration or structure of ergosterol. He also informed me that he had finally proved that the newly discovered sulphur atom in vitamin B is present as a sulfo-pyrrol grouping, but he was not certain whether the sulfur was present as a double-bond sulfur atom or whether it was present as sulfhydryl. This is of particular interest to biologists and biological chemists for the reason that it suggests a number of biological questions regarding the possibility of the function of vitamin B in the living cell. It is entirely possible that vitamin B may owe its properties to the oxidative-reductive properties of the sulphur present.

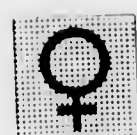
If space permitted I should like to say something about the fine research work that is being conducted at the University of Halle in the field of soils and crops; of the work of Professor Neubauer of Dresden on the utilization of nutrients by plants; of the fine work on the chemistry of soils and crops and the work of the dairy research institute at Weihestephau; of Dr. Koenig's brilliant work on tobacco at Forchheim; of the brilliant work of Warburg and Neuberg in their respective institutes at Berlin-Dahlem and other interesting researches too numerous to mention.

I cannot close this paper without mentioning the very fine Hygienic Museum at Dresden which no biologist or chemist can afford to miss. This is probably the best museum of its type in the world. No chemist should make a trip to Germany without visiting the National Museum at Nurnberg, and the enormous Technical Museum at Munich. These museums contain reproductions of old alchemical laboratories as well as old apothecary shops. I spent most of one day at the old Liebig Museum at Giessen where Liebig spent so many years, and where he made his reputation as an agricultural chemist. His old laboratories and lecture room have been preserved just as they were when he became Director of the Institute as a young man just out of the university.

In closing I might say that I found but one type of research which had been directly affected by Hitler personally. Hitler does not use tobacco or alcohol in any form and he is discouraging the use of alcohol and tobacco by German youth. In their enthusiasm for their

leader, the young people of Germany are endeavoring to be as much like him as possible, with the result that it is rather unusual to see German young people consuming wine or beer. This attitude on the part of German youth has not only had its effect on the consumption of alcoholic beverages, but it has stimulated research work on the unfermented fruit juices which are now being sold in Germany in increasing amounts. It is now possible to obtain unfermented fruit juices made from the apple, strawberry, cherry, raspberry and other fruits which have been made enzyme- and bacteria-free by special methods of filtration. As a result these fruit juices do not have to be pasteurized and consequently have retained most of their fruity flavor. These beverages are very popular throughout Germany at the present time.

While we have progressed very rapidly in research and actually surpass Germany in many lines of work, I feel that the chemical student can still visit Germany with considerable pleasure and profit. The scientist will always be received in Germany with courtesy and hospitality, for science knows no politics and recognizes no national boundaries.



SOME RECENT TRENDS IN VITAMIN RESEARCH¹

R. ADAMS DUTCHER

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THE invitation to address your annual meeting came to me in Berlin last July just as I finished a five month scientific pilgrimage to many of the research laboratories of Germany and neighboring countries. I have chosen to discuss a number of different phases of pure and applied research which in my opinion indicate the direction in which vitamin investigations are pointed at the present time.

The past two years have been most productive in several lines of work, particularly with regard to the application of organic chemistry and physics to the solution of fundamental problems of vitamin determination, isolation, purification and configuration. Much excellent work has also been done on the clarification of questions regarding vitamin function and further steps taken to arrive at workable international standards of vitamin unitage.

Commercialization of vitamins has increased at a surprising rate. For the first time in history a vitamin has been synthesized in the laboratory and, in the course of a few months, has reached a stage of commercial production where the manufacturer speaks of its yield in terms of kilograms and pounds instead of grams and ounces.

With improved methods and techniques and with the use of natural and artificial anti-oxidants, vitamin concentrates are being increased in potency and stability. Foods of many types are being fortified and supplemented with vitamins in various forms and by various methods.

The pediatrician, dietitian, food control official and nutrition worker are confronted with new and baffling problems. Questions are raised by laymen as well as scientists regarding the respective merits of natural and artificial proprietary vitamin sources for various purposes and needs. Not until the facts revealed by animal experiments can be more intelligently translated into human values will it be possible to evaluate from a quantitative standpoint much of the excellent work going on at the present time. Sufficient work has been done with the higher animals and also in hospitals and clinics to know that many, if not most of the conclusions drawn from experiments on laboratory animals can be applied in principle and in a qualitative way to human beings and to domesticated live stock. Only occasionally do we find that the higher animals

¹ Presented before the American Dietetic Association, Washington, D. C., October 17, 1934.

deport themselves in a physiological manner which differs fundamentally from that of the lower animals.

The response of the medical profession to the work of those in the field of small animal experimentation, and the extent of the clinical research work projected with the view of clarifying many questions which have awaited translation into terms of human health and welfare, is gratifying.

A similar tendency has manifested itself in the field of domestic animal experimentation and practical feeding. We are beginning to find that the veterinary profession is able to utilize the findings of small animal research in the alleviation of many of the nutritional disturbances in domesticated animals and in wild animals in captivity. Farmers and scientists, who deal with expensive and highly bred live stock, have utilized many of the discoveries which originated in studies with small animals, and have made interesting scientific and practical applications in the live stock field.

The desire for the application of fundamental laboratory discoveries is so great at the present time that an announcement of a new discovery is followed almost immediately by clinical, industrial and field tests along practical lines.

It is equally significant that chemical and biological laboratories can now be found in many industrial manufacturing plants in almost every civilized country of the world. Manufacturers of many foods and of biological products now maintain their own vitamin testing laboratories, and where five years ago there were in this country but two or three commercial consulting laboratories equipped to conduct vitamin testing, there are now several of these laboratories scattered from the Eastern seaboard, through the Middlewest to the Pacific coast.

A few state bureaus of food and feed control have already established their own vitamin testing laboratories and all signs point to the necessity of other states following their example in the not distant future.

These it seems to me are some of the general trends and tendencies which are the direct result of vitamin research of the past few years. We will give a brief résumé of a few of the actual research problems of the past two years which are certain to influence and modify our ideas regarding foods, food values and dietetic practices.

VITAMIN A

In 1927, H. von Euler of Stockholm established the fact that pure carotene ($C_{40}H_{56}$) produced, in vitamin A-deficient rats, a type of physi-

ological response which seemed to be identical with that produced when vitamin A concentrates are fed. This work was followed shortly by the researches of T. Moore of England, who not only verified the work of von Euler but proved in a most interesting and conclusive manner that carotene is catabolized in the tissues with the formation of vitamin A. As a result of these and subsequent researches, carotene is now recognized as a precursor of vitamin A and referred to as a "provitamin."

Brilliant researches on the plant pigments by R. Kuhn of Heidelberg and by Paul Karrer of Zurich have done much to clarify our ideas regarding the properties and configuration of the carotenes and of vitamin A.

Kuhn, collaborating with Brockmann at Heidelberg and with A. Scheunert at Leipzig, has concluded that ordinary crystallized carotene often contains three isomeric carotenes (known as α , β and γ carotenes) all of which possess identical qualitative effects on growth, xerophthalmia and the sexual cycle. From a quantitative standpoint, however, β carotene is about twice as potent as the α and γ forms.

It is quite clear that β carotene consists of two β ionone rings united by a series of conjugated double bonds. The isomeric carotenes differ only in the configuration of the terminal structures.

Karrer has established the configuration of vitamin A and all evidence points to the fact that two molecules of vitamin A are formed from one molecule of carotene, while the unsymmetrical α and γ forms yield but one molecule of vitamin A. When I was in Karrer's laboratory last May, he informed me that he was attempting to synthesize vitamin A in the laboratory but that the problem was fraught with many difficulties. He stated that he had been able to add some of the double bonds to the polyene chain but that he had been unable to add the terminal unsaturated groupings, owing to the instability of the chain.

Kuhn states that he is convinced that six provitamins-A are known, the naturally occurring carotenes referred to above and three derivatives of β carotene: β oxy-carotene, β carotene oxide and semi- β -carotenone. To my knowledge the above represent the most recent trends so far as the chemistry of vitamin A and the carotenoid pigments is concerned.

Evidence has accumulated which throws further doubt on the clinical value of vitamin A as a general anti-infective agent. While it is an established fact that membrane tissues show a characteristic breakdown in vitamin A deficiency, it is doubtful if clinical evidence exists to show that tissue recovery, after vitamin A therapy, bears a definite relationship to cessation of clinical infection. From a theoretical standpoint heavily

secreting normal membrane tissues should offer less opportunity for bacterial invasion. Conversely, tissue breakdown followed by lessened mucous secretion should offer a better opportunity for infective organisms to obtain a foothold.

The discovery that carotene contributes to the vitamin A potency of foods has stimulated many other types of research, one of which is the part played by carotene in the vitamin A potency of butter fat. Baumann and Steenbock at the University of Wisconsin are of the opinion that 85 per cent of the total vitamin A potency of butter fat is due to vitamin A, *per se*, while but 15 per cent is due to the pigment carotene. This is in close agreement with the findings of Kon of England who informed me that his data indicate that the amount of vitamin A is approximately seven times that of the carotene ordinarily present in normal, well colored butter fat.

It is a matter of common observation that breeds of dairy cattle can be classified according to their ability to produce yellow butter fat. The Guernsey and the Jersey breeds are renowned for their ability to produce a highly pigmented butter fat, while Holstein, Ayrshire and Shorthorn breeds produce a butter fat which is much less rich in color, in spite of the fact that the various breeds may have been fed identical dairy rations.

It was a natural consequence that breeders of the so-called "pigmented" breeds of dairy cattle should ascribe added nutritional virtues to the carotene-rich milks, just as soon as research laboratories began to emphasize the fact that carotene had the properties of vitamin A.

During the past two years a number of laboratories have attempted to answer the question: "Does one dairy breed possess physiological powers not possessed by another dairy breed so far as vitamin A excretion is concerned?"

Sutton and Krause of Ohio concluded that Holstein butter fat was richer in vitamin A than Guernsey butter fat, in spite of the fact that the latter is richer in carotene. They found that the carotene content of butter fat from the various breeds was in the ascending order: Ayrshire, Holstein, Jersey and Guernsey. While pasture feeding enhanced the color of butter fats of all four breeds, the relative order of color values remained the same.

Hathaway and Davis of Nebraska have studied the same problem, but have used standardized creams as their source of carotene and vitamin A. They concluded that the total vitamin A potency of Holstein cream is somewhat higher than that produced by the Guernsey breed.

Wilbur, Hilton and Hauge of Indiana could detect no significant nutritional differences when butters from Holstein and Guernsey cattle were tested for vitamin A content. Similar results have been reported by Kon and Booth and by Watson, Bishop, Drummond, Gillam and Heilbron of England.

At the Pennsylvania State College, Guerrant, Harris and I have been studying this problem with the coöperation of Bechdel and Shaw of the Department of Dairy Husbandry. In our work we have departed from the procedures described above; instead of feeding butter, butter fat or cream we have used fresh milk, fed at various levels. Our investigation has been under way for two years and the work has been attended by a feeling of uncertainty owing to the pronounced variability among individuals of the same breed. At the present time our results do not seem to show significant breed differences, so far as the total vitamin A potency of Holstein and Guernsey milks is concerned, when data are calculated on the basis of the vitamin A units per gram of butter fat. When data are considered solely from the standpoint of milk potency, there is evidence that the Guernsey milks are appreciably superior.

It is evident that the "poorly pigmented" breeds (such as the Holstein and Ayrshire) possess a very efficient carotene-converting mechanism with the result that they excrete less carotene in the milk and more of the colorless vitamin A. On the other hand, it is probable that the Guernsey and Jersey breeds store more carotene in their tissues and excrete more of this pigment in their milk because of their inability to catabolize large amounts of this pigment. If this is true, Guernsey and Jersey milks should owe a larger proportion of their vitamin A potency to carotene and a smaller proportion to vitamin A, *per se*, than is the case with the Holstein and Ayrshire milks. We hope to find an answer to this question.

We have finally proved to our own satisfaction that carotene is not assimilated satisfactorily by the rat when this pigment is fed in the presence of mineral oil. When butter fat is fed at "threshold levels" as the sole source of vitamin A, animals will live, maintain body weight or grow slowly and show no signs of xerophthalmia. When the same levels of butter fat are fed in the presence of small amounts of mineral oil, the animals die with the usual symptoms of vitamin A deficiency. Similar results are obtained when pure carotene is fed as the source of vitamin A. When non-pigmented cod liver oil is fed in the presence of mineral oil, assimilation of vitamin A seems to be quite satisfactory. When mixtures of carotene and mineral oil are fed at various levels, pigment ex-

cretion in the feces increases as the carotene dosage becomes larger. We have attempted to explain these results on the theory that the unassimilated (hydrocarbon) mineral oil possesses a preferential solubility for the hydrocarbon carotene, while the lipoidal or sterol containing intestinal fluids possess a preferential solubility for the sterol vitamin A, thereby removing vitamin A from the unabsorbed mineral oil.

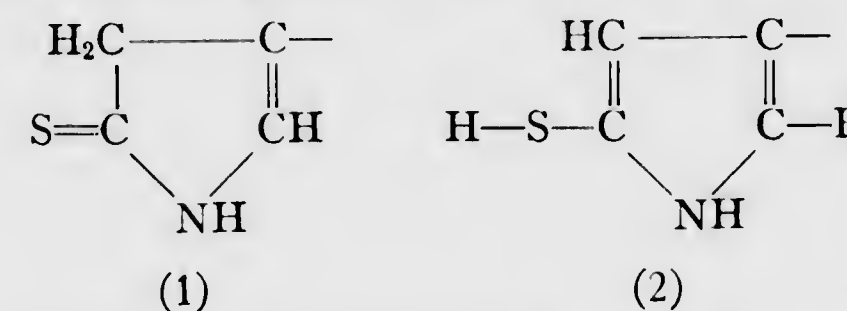
These findings are largely of academic interest. Human beings who use mineral oil as an intestinal lubricant have little reason to be concerned for the reason that mineral oil is usually ingested several hours after the meal has digested and after considerable absorption of nutrients has taken place. It is possible that salad dressings made of mineral oil may prevent some carotene utilization but these, I am informed, are sold to a very limited extent.

VITAMIN B (B₁)

An increasingly large literature on the B vitamins has accumulated during the past two years. It seems evident that some of the crystalline preparations of vitamin B (B₁) which have been described by Jansen and Donath, Peters and coworkers, Windaus, and Ohdake are identical substances, although workers are not yet agreed that these crystalline products are completely uncontaminated.

Windaus of Germany, Ohdake of Japan and Van Veen of Batavia have shown that crystalline vitamin B preparations all contain sulfur and this has been confirmed by other workers. Most workers are agreed that the molecule contains twelve carbon atoms and one sulfur atom, but the other atomic constituents have not been established with certainty. The following formulae have been suggested: Windaus, C₁₂H₁₇ON₃S; Van Veen, C₁₂H₁₆O₂N₄S; Ohdake, C₁₂H₂₀O₂N₄S.

Windaus of Gottingen informed me last July that he had good evidence that the sulfur present in the vitamin B molecule is in the form of a sulfo-pyrrol. He is inclined to believe that the sulfur atom is combined as in (1) but stated that it is possible that formula (2) may be correct.



While it is dangerous to speculate regarding the relation of configuration and constitution to physiological action, one wonders whether or

not this vitamin may owe some of its properties to the oxidative-reductive properties of the sulfur present.

Peters and coworkers at Oxford have contended that the oxygen uptake of brain tissue of pigeons afflicted with vitamin B deficiency is lower than that of normal brain tissue. Theories are advanced that this vitamin may act as a co-enzyme in an oxidative enzyme system, but the whole problem is affected by so many variables that I merely call attention to its interesting potentialities.

VITAMIN G (B₂)

Prior to 1933, little progress had been made relative to the chemistry of vitamin G (B₂). In that year Richard Kuhn and associates of Heidelberg made the interesting announcement that the so-called lyochromes or flavine pigments had been isolated in crystalline form and that these pure crystalline products seemed to possess properties similar to if not identical with vitamin G or B₂. These flavines have been prepared from milk whey (lacto-flavine), liver (hepo-flavine) and eggs (ovoflavine). Kuhn informed me that (after three crystallizations) it was possible to obtain 50 milligrams of ovoflavine from 50 kilograms of dried egg albumin, which represents the albumin from about 17,000 eggs. From 3000 liters of cows' milk he was able to obtain 60 milligrams of lacto-flavine.

A chemical study of these flavines indicated that they all have the empirical formula $C_{17}H_{20}N_4O_6$. Absorption spectra of the different flavines were practically identical and mixtures of the crystals from different sources gave no depression of the melting point.

He found that a portion of the molecule, containing hydroxyl groups, could be split off by irradiation. When unirradiated crystals were fed to rats which had been brought to a state of vitamin G deficiency, growth response ensued, providing vitamin B₄ was present. Kuhn and his coworkers have been unable to produce the pellagra-like dermatitis in their rats and consequently no data are available regarding the anti-dermatitis properties of the flavines.

The Heidelberg workers have shown that Warburg's yellow enzyme possesses vitamin G activity and they were able to separate the active component from its colloidal carrier and identified the active component as a flavine. The flavines disintegrate when exposed to ordinary light, with the formation of a chloroform-soluble fragment which has the formula $C_{13}H_{12}N_4O_2$. For this reason it is necessary to protect these

products from ordinary light by using yellow or brown desiccators. Jansen and his colleagues at Amsterdam had a laboratory specially set aside for these products and all windows were painted yellow to absorb the harmful or destructive blue and violet light rays.

A well known English worker informed me that the flavines had been tested in an English laboratory and that it is possible to reproduce the growth responses in rats as described by Kuhn but that the flavines have little or no effect on the prevention or cure of the pellagrous type of dermatitis. This worker also informed me that she questioned the importance of the B₄ arguments of Kuhn, owing to the fact that the Peters fractions used as a source of B₁ in these experiments always carry ample amounts of vitamin B₄.

Kuhn's observations have opened up a number of biological problems in the fields of animal nutrition, cell respiration and the mechanism of enzyme action.

In our laboratory, Guerrant has continued his study of the synthesis of vitamins B and G in the digestive tracts of coprophagous rats. His studies of the past few months have shown that the type of carbohydrate in the diet is a very important factor. When dextrinized starch is the sole carbohydrate, it is possible for the coprophagous rat to synthesize these vitamins. When sucrose is the sole source of carbohydrate, coprophagous rats die with the usual symptoms of B and G avitaminosis. The study is being continued with other carbohydrates to find an explanation for these findings.

VITAMIN C

It would appear that the final chapter has been written relative to the chemistry of vitamin C. It has been synthesized and its commercial production as ascorbic acid is now a matter of record.

The early work of Zilva of England laid the ground work for the isolation and identification of this vitamin when he devised chemical methods for the chemical fractionation of lemon juice. By the use of these methods it was possible to remove the citric acid, sugars and other extraneous materials and obtain a very active antiscorbutic concentrate by precipitation with lead acetate.

As early as 1928, Szent-Gyorgyi of Hungary had called attention to the fact that he had evidence for the existence of a reducing substance (hexuronic acid) in lemon juice, cabbage juice and in various other plant and animal tissues which seemed to be similar to vitamin C so far as

distribution in food materials is concerned. For a time Zilva did not accept the theory that reducing capacity and antiscorbutic properties might be correlated.

In the meantime, King and coworkers at the University of Pittsburgh had succeeded in isolating a crystalline product from lemon juice which showed high antiscorbutic activity and which possessed all of the physical and chemical properties of Szent-Gyorgyi's hexuronic acid.

In 1932, Szent-Gyorgyi (with Svirbely, who had worked previously with King) announced that their crystalline hexuronic acid possessed antiscorbutic properties and that vitamin C and hexuronic acid were undoubtedly identical substances.

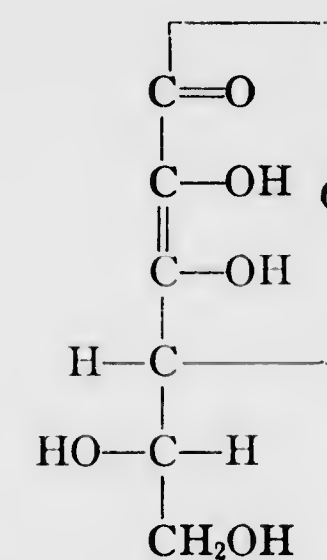
Tillmans of Germany and his colleagues concluded that hexuronic acid and vitamin C are identical and devised in their laboratory a useful titration method (based on the reduction of the dye 2,6 dichlorophenol-indophenol). The Tillmans' method has since been modified by King and coworkers with the result that it is now possible to measure, with a fair degree of accuracy, the vitamin C content of many foods without recourse to tedious and expensive biological assays with guinea pigs. We have used this method with considerable success in our own laboratory in assaying fruit and vegetable juices for vitamin C. Our titration data on these juices and on cow's milk agree remarkably well with biological data obtained simultaneously.

Shaw, Rasmussen, Guerrant, Welch and Bechdel of the Pennsylvania State College have used the titration technique in a study of the vitamin C content of milks from various breeds. While it is too early to draw definite conclusions, it would appear that the vitamin C potencies of milks from the various breeds run in the ascending order: Holstein, Ayrshire, Guernsey, Brown Swiss, Jersey.

Final evidence regarding the identity of vitamin C was afforded by the researches of Haworth and coworkers at Birmingham, Karrer and colleagues at Zurich, von Euler and Martius at Stockholm, and others, that vitamin C (now known as ascorbic acid) is 3-keto-*l*-gulo-furano-lactone with the formula on page 485.

While in Switzerland I learned that ascorbic acid is now being synthesized in commercial quantities in a chemical manufacturing plant in Basle. The method of manufacture is entirely synthetic and depends on the method devised and patented by Reichstein of Zurich: the oxidation of *l*-sorbose. We have made chemical and biological tests of this synthetic ascorbic acid and have found it to be extremely active. Steps

are now being taken to make ascorbic acid the official international standard for vitamin C.



Ascorbic acid

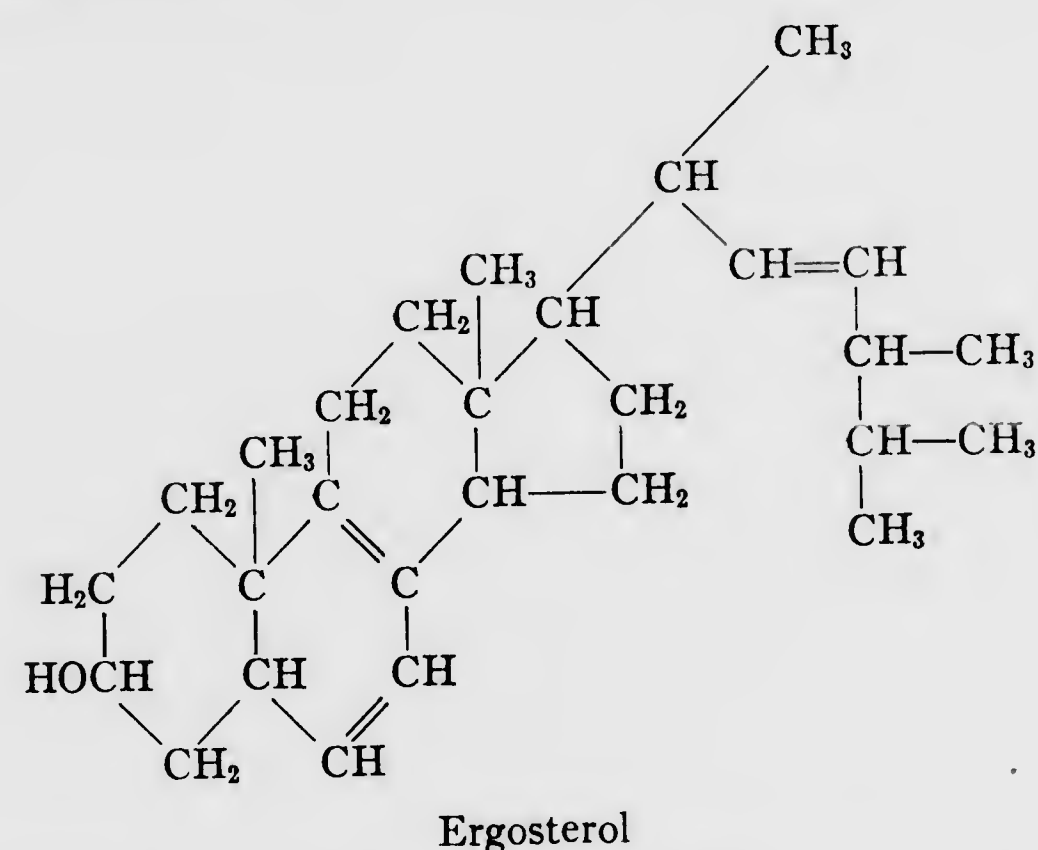
A well-known manufacturer of chemical and biological products in Germany is also manufacturing relatively large quantities of ascorbic acid. I understand that his product is natural ascorbic acid, obtained by the extraction of peppers and similar plant materials. Tillmans and his group have shown that the seed pods of rose plants give excellent yields of ascorbic acid.

Stepp at the Medical Clinic in Munich informed me that clinical experiments were under way at his clinic which offered encouraging evidence that ascorbic acid might be used to advantage in certain diseases which are characterized by skin pigmentation, particularly Addison's disease. Purr of the Cancer Research Laboratories of the Graduate School of Medicine at Philadelphia recently obtained evidence which led to some interesting and important hypotheses with reference to the probable function of vitamin C in the activation of intracellular enzyme systems in normal and pathological tissues. Harris at Cambridge University has made the significant observation that ascorbic acid is found in appreciable quantities in pea seeds just as soon as germination begins, while S. N. Ray from the same laboratory has shown that hen's eggs contain no vitamin C but that this vitamin appears in the chick embryo as early as four days after incubation.

VITAMIN D

No outstanding advances relative to the chemistry of vitamin D have been made since the discovery that activated ergosterol can be prepared in crystalline form. Considerable progress has been made with

regard to the determination of the probable structure of ergosterol, but to date we have no clear idea of the structural changes which occur when inactivated ergosterol is changed to the active form by irradiation. The following configuration of ergosterol has recently been advocated by Windaus and coworkers.



A considerable literature has been published in the past two years relative to the toxicity of ergosterol preparations and the probable physiological effects of over dosage. One of the most comprehensive studies in this field is that of Hauch at the University of Chicago. This writer reared rats of varying ages on a stock diet containing 50,000 times the curative dose of irradiated ergosterol, without observing harmful effects. Young rats were able to live for one year on the stock ration when the dosage was 93,000 times the curative dose. Adult rats, however, were not able to withstand doses of this magnitude. This author concluded that toxicity was less marked when the diet was complete and well balanced.

Evidence continues to accumulate which indicates that vitamin D may exist in two or more forms. Kon and Booth of the National Institute for Research in Dairying at Reading, England, have shown that about 80 per cent of the antirachitic potency of butter fat is lost after saponification, while activated ergosterol and cod liver oil, either alone or mixed with butter, can be subjected to saponification without loss of potency. They find that most of the antirachitic potency is present in the fatty acid or saponifiable fraction when losses, such as those just

described, have occurred. They found that a greater proportion of vitamin D activity remained in the unsaponifiable portion of butter fat when the antirachitic potency of the butter fat was increased by irradiating the butter or by feeding irradiated yeast to the cows.

Rygh of Oslo, Norway, has reported similar observations. He has confirmed not only the findings of Kon and Booth on butter fat but he has conducted similar experiments with ether soluble residues obtained from meadow hays and human and cow's liver. In every case a portion of the antirachitic potency could be found in the fatty acid fraction, indicating that we may be forced to recognize more than one antirachitic factor.

It is common knowledge that the chicken has a vitamin D demand which seems to be many times that of the rat. Bethke and coworkers at the Ohio Experiment Station and others are convinced that many more rat units in the form of ergosterol, than in cod liver oil, must be used to bring about a given physiological response in chickens.

Within the past few months J. Waddell has submitted evidence from which he concludes that cholesterol contains a provitamin which is not ergosterol. By the activation of this provitamin with ultra violet light he has obtained a substance which is as potent for chicks as the vitamin D of cod liver oil. Waddell's work lends additional support to the growing conviction that the antirachitic properties of foods may be due to more than one chemical substance.

R. R. Murphy and Hunter, in my laboratory, coöperating with Knandel of the Department of Poultry Husbandry, have made a very thorough study of the vitamin D requirements of growing chicks and laying hens. Their results indicate that the growing pullet requires a yearly intake of about 25,000 International units of vitamin D when cod liver oil is the sole source of this vitamin.

Clinical evidence is woefully lacking regarding the merits and demerits of the various types of vitamin D milks. I am inclined to take the stand that evidence is lacking, as yet, to show that one form of vitamin D is better utilized than another. Even if such evidence is eventually available, I see no reason why one source of vitamin D should be considered better than another, if a proper standard for each source of vitamin D can be agreed upon by medical and nutritional authorities. This is a matter which deserves the immediate attention of medical and food control officials.

Certainly it is unnecessary and unwise to supplement milk with vitamin D beyond that point where milk will contain sufficient antirachitic

potency to assure the necessary protection of normal children during the winter months. When higher doses of vitamin D are required the matter should be referred to the family physician.

The matter of unitage is a puzzling problem to every one concerned with vitamin D therapy, and much remains to be done to clarify the confused situation that exists at the present time. Table 1, devised by Lachat and Halverson of the Minnesota Division of Feed Control, is most helpful in evaluating the various vitamin D units now in use.

A few years ago a young graduate student remarked to me that since vitamin research had made such rapid progress it seemed to him most of the important problems had been solved and little more remained to

TABLE 1
Vitamin D rat unit equivalents

	INTER- NATIONAL UNITS	U.S.P. UNITS	STEEN- BOCK UNITS	AM. MED. ASSN. UNITS	AM. DRUG MFRS. ASSN. UNITS	ONE D UNITS	OSLO UNITS
One International unit equals....	1.00	0.37	0.37	3.25-3.70	0.028	1.67
One U.S.P. unit equals.....	1.00	0.37	0.37	3.25-3.70	0.028	1.67
One Steenbock unit equals.....	2.70	2.70	1.00	10.00	0.075	4.50
One Am. Med. Assn. unit equals..	2.70	2.70	1.00	10.00	0.075	4.50
One Am. Drug Mfrs. Assn. unit equals.....	0.27	0.27	0.10	0.10	0.008	0.45
One D unit equals.....	36.00	36.00	13.33	13.33	133.30	60.00
One Oslo unit equals.....	0.60	0.60	0.22	0.22	2.22	0.017

be done. Since that time thousands of research papers have been published in various phases of vitamin research; yet every new discovery seems to create many more new problems of even greater importance and significance. No one can prophecy what the next two years of research will bring forth but I am convinced that they will be even more productive and valuable than the past two years have been.

My opinion is that the most outstanding trends during the past two or three years have been in the direction of vitamin isolation, purification, synthesis, the determination of configuration, and the application of improved chemical, physical and biological methods to the clarification of fundamental questions related to the physiological function of vitamins.

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THE VALUE OF THE CHEMICAL TITRATION METHOD IN DETERMINING THE VITAMIN C POTENCY OF CERTAIN FOOD SUBSTANCES¹

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Recent investigations have shown that vitamin C and ascorbic acid are identical substances (King and Waugh, '32 a and b; Harris and Ray, '33; Herbert, Hirst, Percival, Reynolds and Smith, '33; Reichstein, Grussner and Oppenauer, '33; Ault, Baird, Charrington, Haworth, Herbert, Hirst, Percival, Smith and Stacey, '33) and that ascorbic acid can be estimated quantitatively by titrating with a standard solution of iodine or a standard solution of 2, 6-dichlorophenol-indophenol.

A review of the literature relative to the chemical estimation of vitamin C in foodstuffs (Tillmans and Hirsch, '32; Wolff, Kekeln and Emmerie, '33; Birch, Harris and Ray, '33; Bessey and King, '33) indicated that the method proposed by Bessey and King is the least affected by interfering substances; consequently, this method was used in the following studies.

The present investigation was conducted with the view of attempting to establish further correlations between chemical titration methods and the usual biological method for the estimation of vitamin C in fruit and vegetable juices.

¹Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station, December 6, 1934, as technical paper no. 669.

EXPERIMENTAL

The studies herein reported consist of the determination of the ascorbic acid content of several fruit and vegetable juices by the chemical method and the subsequent comparison of some of the results obtained by the conventional biological method of vitamin C assay.

Chemical assay

Bessey and King ('33) have pointed out that lemon juice could be used with reasonable accuracy in the standardization of the dye, while Birch, Harris and Ray ('33) stated that both lemon and orange juice are reliable in this respect.

In these studies, lemon juice was always used in the standardization of the dye solution. The technic used is as follows: The dye solutions were prepared just previous to being used by dissolving 0.1 gm. of the dye in successive portions of warm water, diluting to 200 ml., then cooling and filtering. A 5 ml. aliquot of the freshly prepared and strained lemon juice was titrated with 0.01 normal iodine solution until a permanent bluing of the starch indicator resulted. Ten milliliters of an 8 per cent acetic acid solution were added to a second 5 ml. aliquot of the lemon juice and the dye solution run in from a burette until the end point (pink) was reached. By means of this technic, interfering substances which fade the dye slowly are easily detected when the end point has been reached.

Since 1 ml. of 0.01 normal iodine solution reacts with 0.88 mg. of ascorbic acid, the ascorbic acid equivalent of the dye solution was easily calculated. Other juices were then evaluated as to their ascorbic acid content by titrating against this dye solution of known titer.

For the purpose of reference, the dye solutions used were standardized occasionally against pure ascorbic acid as well as against several different fruit juices. In table 1 are given the results obtained by standardizing some of the dye solution against ascorbic acid, lemon juice, orange juice, and grapefruit juice. The results are expressed as milligrams of ascorbic acid for each milliliter of the dye solution.

The above results indicated that lemon juice and grapefruit juice could be used as standards without introducing serious errors in the technic. Orange juice, however, appeared to be less reliable in this respect. While the juice of only one orange was titrated in direct comparison with a known amount of ascorbic acid on each of the above days, other titrations of orange juice showed about the same irregularities when lemon juice was used as the check standard.

Biological assay

The biological assays were carried out according to the technic of Sherman, LaMer and Campbell ('22). Young guinea pigs weighing from 250 to 300 gm. were used as the

TABLE 1
Showing the results obtained by standardizing some of the dye solutions against ascorbic acid, lemon juice, orange juice and grapefruit juice

DATE	ASCORBIC ACID STANDARDIZATION	LEMON JUICE STANDARDIZATION	ORANGE JUICE STANDARDIZATION	GRAPEFRUIT JUICE STANDARDIZATION
4/25/34	0.147 ¹	0.155	0.165
5/24/34	0.149 ²	0.150	0.160	0.150
6/26/34	0.144 ³	0.152	0.146

¹ Natural ascorbic acid furnished through the courtesy of Dr. C. G. King.

² Natural ascorbic acid furnished through the courtesy of Merck & Co.

³ Synthetic ascorbic acid furnished through the courtesy of Hoffmann-LaRoche & Co.

experimental animals. These pigs were kept in individual metal cages which were provided with raised screen floors. With the exception of a slight modification of the scorbutic diet, these animals were fed and handled according to the technic of the above authors. The basal scorbutic diet used in these studies contained 2 per cent of cod liver oil (Patch's medicinal grade) and 8 per cent of butter fat instead of the usual 10 per cent of butter fat. This alteration in the composition of the scorbutic diet was made in order to insure a liberal supply of vitamin D.

The first series of biological assays for vitamin C were made on fresh Florida orange juice and canned pineapple juice. A week's supply of the fresh fruit and the canned juice

was kept in the refrigerator at all times during the test period. The juice of the oranges was extracted just previous to titration and feeding. The canned pineapple juice was opened just before being used.

Shrader and Johnson ('34) and Nelson and Molten ('34) had shown that when fruit juices are kept frozen for long periods of time, there is no appreciable loss of antiscorbutic potency. Consequently, for the second series of biological assays, composite samples of the various juices were preserved in the above manner until the assays could be made. In the case of lemon juice, 5 dozen of each of three commercial grades of lemons were used as the source of juice. One dozen select grapefruits and 1 dozen fresh pineapples were used as sources of these juices. The fruits were pared by hand and the juices pressed out mechanically. The juices were strained and placed in small waxed-cardboard containers, each of which held an ample supply for daily experimentation, and then stored at a temperature of -10°F . The freshly thawed juices were fed to guinea pigs on the basis of the calculated minimum protective dosage as determined by the dye titer. It was hoped that by such a procedure, a uniform supply of the various juices could be had for the entire experimental period, and that results could be obtained which would show whether or not the calculated levels of these juices furnished the desired degree of protection to guinea pigs, and, further, that some information might be obtained concerning a possible variation in the antiscorbutic potency of juices from different grades of lemons. The stored juices were titrated from time to time during the storage period against a standard dye solution. The resultant titers showed that no marked decrease in vitamin C potency took place during the 90-day storage period.

Bessey and King ('33) have shown that 0.5 mg. of ascorbic acid daily per guinea pig prevented scurvy, allowed satisfactory survival, and maintained the experimental animals in a condition of reasonable sensitivity to changes in vitamin intake. Therefore, in these studies the calculations have been based on the assumption that 0.5 mg. of ascorbic acid or its

equivalent is the minimum protective dose for the average size experimental guinea pig.

DATA AND DISCUSSION

A condensed summary of the data obtained in these studies is given in the following tables.

Table 2 gives the results obtained in a series of biological assays that were made on canned pineapple juice and fresh

TABLE 2
Showing the results obtained by feeding varying amounts of canned pineapple juice and fresh orange juice to groups of young guinea pigs that were receiving a scurvy-producing diet

ANIMAL GROUP NO.	NUMBER OF ANIMALS CONSIDERED	SOURCE OF VITAMIN C (JUICES)	QUANTITY OF JUICE FED DAILY	TITRATION EQUIVALENT IN MILLI-GRAMS OF ASCORBIC ACID	THEORETICAL ¹ INTERNATIONAL UNITS	AVERAGE DAILY GAIN IN WEIGHT	SCURVY SCORE
			ml.			gm.	
1	6	Pineapple	0	0	0	All died	Severe
2	6	Pineapple	3.0	0.245	5.7	All died	Severe
3	3	Pineapple	4.0	0.352	7.6	0	17
4	4	Pineapple	5.0	0.440	9.5	0.5	8
5	9	Pineapple	6.0	0.528	11.4	1.8	3
6	7	Pineapple	7.0	0.616	13.3	2.4	0-2
7	5	Pineapple	8.0	0.707	15.2	2.7	0
8	8	Pineapple	9.0	0.792	17.1	2.7	0
9	7	Pineapple	10.0	0.880	19.0	2.9	0
10	4	Orange	1.0	0.500	11.0	2.8	0-2
11	9	Orange	1.5	0.750	16.5	3.0	0
12	9	Orange	2.0	1.000	22.0	3.2	0
13	8	Orange	3.0	1.500	33.0	3.2	0
14	6	Orange	4.0	2.000	44.0	3.6	0
15	5	Orange	5.0	2.500	55.0	3.7	0

¹ Calculations based on the average value (1.1 ml. = 0.5 mg. of ascorbic acid) obtained by titrating ten different samples of lemon juice.

orange juice. The table also contains the equivalent ascorbic acid content of the respective quantities of the two juices fed, as determined by the dye titration method. In addition, the theoretical International units of vitamin C are given.

The six animals comprising group 1, which served as a negative control, were all dead by the thirty-first day, while those animals comprising group 2 were all dead by the forty-second day. On autopsy, all animals of these two groups were

found to exhibit symptoms of severe scurvy. Although the animals of groups 3 and 4 survived the experimental period, they likewise showed symptoms of severe scurvy. The animals of group 5 were somewhat irregular in their responses and, when autopsied, their scurvy score ranged from 1 to 6, with an average score of 3.

The animals of group 6 showed only very mild symptoms of scurvy and their growth rates were not significantly depressed. Due to these facts, this group of animals was considered as having received a minimum protective dose of vitamin C or the equivalent of 0.5 mg. of ascorbic acid daily.

The animals of groups 7, 8 and 9 all showed practically the same growth rate and survived the experimental period without showing any evidence of scurvy.

The animals of group 10, which received orange juice, made a similar response to that of those animals comprising group 6, which received the canned pineapple juice. From this it appeared that 1 ml. of fresh orange juice was equivalent to about 7 ml. of canned pineapple juice in antiscorbutic potency. Results obtained by the dye titration had indicated that the relative antiscorbutic potencies of the two juices would be about 6:1.

The animals of groups 11, 12, 13, 14 and 15 showed no evidence of scurvy during the experimental period and when autopsied they appeared to be normal in every respect.

In table 3 are given the results of a second series of biological assays. It may be observed from the data given in this table that the biological responses of the several groups of animals were quite uniform. In every case, with the exception of one group, the calculated dosage gave the expected response. The animals of group 16, which received the calculated sub-protective dosage of fresh pineapple juice, did not show as severe scurvy symptoms as might have been expected from the results given in a previous publication on this subject (Bessey and King, '33).

Table 4 contains the data obtained by titrating the juices of several fruits and vegetables against a standard solution

TABLE 3

Showing the biological responses made by young guinea pigs receiving a scorbutic diet when this diet was supplemented by such quantities of the various fruit juices as would furnish a definite amount of ascorbic acid

ANIMAL GROUP NO.	NUMBER OF ANIMALS CONSIDERED	SOURCE OF VITAMIN C (JUICES)	QUANTITY OF JUICE FED DAILY	TITRATION EQUIVALENT IN MILLIGRAMS OF ASCORBIC ACID	INTER-NATIONAL UNITS	AVERAGE DAILY GAIN IN WEIGHT	SCURVY SCORE
			ml.			gm.	
16	3	Pineapple	1.0	0.3	6.6	2.4	6
17	5	Pineapple	1.6	0.5	11.0	2.4	0-2
18	5	Pineapple	2.2	0.7	15.4	3.4	0
19	4	Lemon I	1.1	0.5	11.0	2.8	0-2
20	3	Lemon II	1.1	0.5	11.0	2.5	0-2
21	5	Lemon III	1.0	0.5	10.0	2.8	0-2
22	6	Grapefruit	1.9	0.5	11.0	2.7	0-2

TABLE 4

Showing the ascorbic acid equivalent and the calculated minimum protective dose of various fruit and vegetable juices as determined by titrating against a standardized solution of 2, 6-dichlorophenolindophenol

KINDS OF JUICE USED	QUALITY OF JUICE	NUMBER OF SAMPLES CONSIDERED	ASCORBIC ACID EQUIVALENT	CALCULATED MINIMUM PROTECTIVE DOSAGE (AVERAGE)
			(mg. per ml.)	(ml.)
Lemon	Fresh	13	0.455	1.1
Orange	Fresh	10	0.500	1.0
Orange	Canned	2	0.294	1.7
Tangerine	Fresh	2	0.208	2.4
Lime	Fresh	1	0.459	1.1
Grapefruit	Fresh	1	0.266	1.9
Grapefruit	Canned	1	0.410	1.2
Pineapple	Fresh	2	0.310	1.6
Pineapple	Canned	18	0.070	7.2
Tomato	Fresh	1	0.157	3.2
Tomato	Canned	1	0.242	2.1
Peach	Fresh	1	0.018	27.8
Plum	Fresh	1	0.030	16.7
Rhubarb	Fresh	1	0.278	1.8
Sauerkraut	Canned	1	0.082	6.2
Cabbage	Fresh	1	0.427	1.2
Peppers	Green	1	0.203	2.5
Carrots	Fresh	1	0.040	12.5
Celery	Fresh	1	0.009	55.6
Turnip	Fresh	1	0.191	2.6
Cucumber	Fresh	1	0.012	41.6
Potato	Fresh	1	0.021	23.8

of the dye. In this table, the results are expressed in terms of the ascorbic acid equivalent in milligrams per milliliter of juice and as the quantity required to furnish a minimum protective dose.

SUMMARY

A study has been made concerning the value of the chemical titration method for determining the vitamin C potency of certain food substances. In these studies, the ascorbic acid content of certain fruit and vegetable juices was determined by titrating against a standard solution of 2, 6-dichlorophenol-indophenol. In order to ascertain the accuracy of the data obtained through the chemical titration method, biological assays were also made on a number of the fruit juices used in this study. From the results obtained it appears that:

1. The chemical titration method for the estimation of ascorbic acid is of material value in determining the vitamin C potency of certain fruit and vegetable juices. By means of this method, it is possible at least to approximate the antiscorbutic activity of the juices before the biological assay is started, thus saving time and expense.

2. The accuracy of the results obtained by the chemical titration method appears to depend to an appreciable degree upon the nature of the juice that is being titrated. Some juices contain interfering substances that react with the dye, thus complicating the titration results and leading to erroneous conclusions.

3. For lemon juice, orange juice, grapefruit juice and fresh pineapple juice, the values obtained by the chemical titration method are in close agreement with those obtained by the usual biological assay method.

4. Fresh pineapple juice, when evaluated for its antiscorbutic potency by the chemical method, gives values in closer agreement with those obtained by the biological method than does the canned pineapple juice, owing to the presence of interfering substances in the latter.

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THE EFFECT OF THE TYPE OF CARBOHYDRATE ON THE SYNTHESIS OF THE B VITAMINS IN THE DIGESTIVE TRACT OF THE RAT*

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In a previous communication from this laboratory (1) data were presented which emphasized the danger of coprophagy as it affects vitamin B and vitamin G assays. In a later publication (2) further data were submitted which showed that the type of carbohydrate employed in making up vitamin B- and vitamin G-deficient dietaries is an important factor in this consideration.

The experiments herein described were carried out as a continuation of the latter investigation. Through these studies, additional data have been obtained concerning dextrinized corn-starch and sucrose-containing diets; and, in addition, diets containing lactose, glucose, and commercial corn-starch have likewise been investigated.

EXPERIMENTAL

The technique employed in this investigation was for the most part similar to that described in a former communication. In the present studies, a series of diets was used which were similar in composition to those previously reported (2). The composition of these several diets differed only in the type of carbohydrate which they contained and is given in Table I.

Young rats obtained from our breeding colony were used as

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experimental animals. These animals were weaned when 21 days of age and were put directly on experiment. In placing the animals on experiment, efforts were made to distribute litters and sexes uniformly throughout the several groups. Each animal was placed in an individual metal cage, which was provided with a raised screen bottom, having 2 meshes per inch. All animals were provided with ample quantities of the respective diets under consideration. Fresh distilled water was kept before the animals at all times, and a weekly record was made of changes in body weight and of the amount of food consumed.

TABLE I
Components and Percentage Composition of Various Diets Used in This Investigation

Components	Diet 349	Diet 353	Diet 355	Diet 356	Diet 357
Extracted casein.....	18	18	18	18	18
Salt Mixture 185*.....	4	4	4	4	4
Agar agar (U.S.P. grade).....	2	2	2	2	2
Cod liver oil (Patch's Medicinal).....	2	2	2	2	2
Butter fat (filtered).....	3	3	3	3	3
Dextrinized corn-starch†.....	71	0	0	0	0
Sucrose (commercial).....	0	71	0	0	0
Lactose (c.p. powder).....	0	0	71	0	0
Glucose (technical).....	0	0	0	71	0
Corn-starch (commercial).....	0	0	0	0	71

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

† Made from commercial corn-starch by moistening the starch with a 0.1 per cent solution of citric acid, autoclaving for 4 hours at 15 pounds pressure, drying, and pulverizing.

The number of animals placed on each of the diets under consideration ranged from seventeen to forty-four animals. In the case of the diet containing dextrinized corn-starch (Diet 349), a total of forty-four animals was used. Twenty-four of these animals (Group 1) received this diet unsupplemented. This group of animals was put on this diet from 17 to 21 days before the remaining animals used in the investigation were placed on experiment. This was done in order that it might be possible to have their feces available as supplements to other vitamin B complex-deficient diets containing types of carbohydrate other than dextrinized corn-

starch. The feces from each of these twenty-four animals were removed from the pans at weekly intervals, combined, dried, extracted with ether, and pulverized. Each weekly collection of feces was treated in a similar manner and added to the previous supply of pulverized feces. Whenever these pulverized feces were fed as dietary supplements, a daily quantity of 0.25 gm. was fed in individual glass containers.

A second group of fifteen animals (Group 2) was placed on the dextrinized corn-starch diet (Diet 349). Beginning with the 2nd day of the experimental period, the total feces voided by each of these animals were placed in separate containers and returned to the respective cages. This practice was continued daily throughout the entire experimental period.

The third group of animals (Group 3) which received this diet was given no additional supplement during the first 21 days of the experiment. On the 21st day all cages were cleaned thoroughly, and beginning with the 22nd day and continuing throughout the remainder of the experimental period, all the feces voided by each of these animals were returned to their respective cages.

A total of twenty-nine animals was fed the sucrose-containing diet (Diet 353). These animals were divided into three groups as follows: Twelve animals (Group 4) received the diet unsupplemented, eleven animals (Group 5) received this diet supplemented daily by all of their own feces, and six animals (Group 6) received this diet supplemented by 0.25 gm. daily of the feces voided by the animals of Group 1.

Seventeen animals received the diet in which lactose constituted the source of carbohydrate (Diet 355). Seven of these animals received the diet without additional supplement (Group 7). Five of the animals (Group 8) had their feces returned to the cage daily, while each of the five remaining animals (Group 9) received daily 0.25 gm. of the feces voided by the animals of Group 1, as a supplement to the diet.

A total of twenty animals received the diet containing glucose as the source of carbohydrate (Diet 356). A group of five of these animals (Group 10) received no further dietary supplement. A second group, containing nine animals (Group 11), had their own feces returned to the cage daily. Each of the remaining six animals (Group 12) received daily allotments of 0.25 gm. of pul-

verized feces which had been voided by those animals composing Group 1.

Eighteen animals received the diet in which commercial corn-starch constituted the source of carbohydrate (Diet 357). Six of these animals received this diet without further supplementation (Group 13). A second group of six animals (Group 14) had their own feces returned to the cage daily, while each of the remaining six animals (Group 15) received daily 0.25 gm. of the pulverized feces from the animals of Group 1, as a supplement to this diet.

A condensed outline of the plan of these studies, together with the corresponding growth responses, is given in Fig. 1.

DISCUSSION

On examining the data obtained in these studies (Fig. 1) it at once becomes apparent that those groups of animals which had access to feces, voided by other animals while receiving a diet in which dextrinized corn-starch constituted the source of carbohydrate, made a much more favorable growth response than did comparable groups of animals which had received the respective diets unsupplemented. This condition is found to be true in every case, regardless of the type of carbohydrate used in the diet. On the other hand, when groups of animals receiving each of the several diets had access to their own feces, marked beneficial effects toward growth were shown only by those animals which had received the diet containing dextrinized corn-starch (Curve 2). A slightly beneficial effect of coprophagy was also shown by those animals which received the diet containing lactose (Curve 8). The well being of those animals which received diets containing sucrose, glucose, or commercial corn-starch was only very slightly affected by having access to their own feces during the entire experimental period (Curves 5, 11, and 14). In fact, the growth responses made by these groups of animals did not differ greatly from the responses made by those groups of animals which received similar dietaries, but which did not have access to their feces (Curves 4, 10, and 13). When the animals composing the various groups were denied access to their own feces, their deportments were found to be relatively similar regardless of the type of carbohydrate used in the diet (Curves 1, 4, 7, 10, and 13), although less paralysis and lower mortality were observed among the animals which received the dextrin diet.

From the above facts it appeared evident that dextrinized corn-starch was at least one of the dietary factors which determines the vitiating effects of coprophagy. Since no plausible explanation was known concerning the mechanism through which this dietary factor reacted in order to bring about this vitiating effect, some

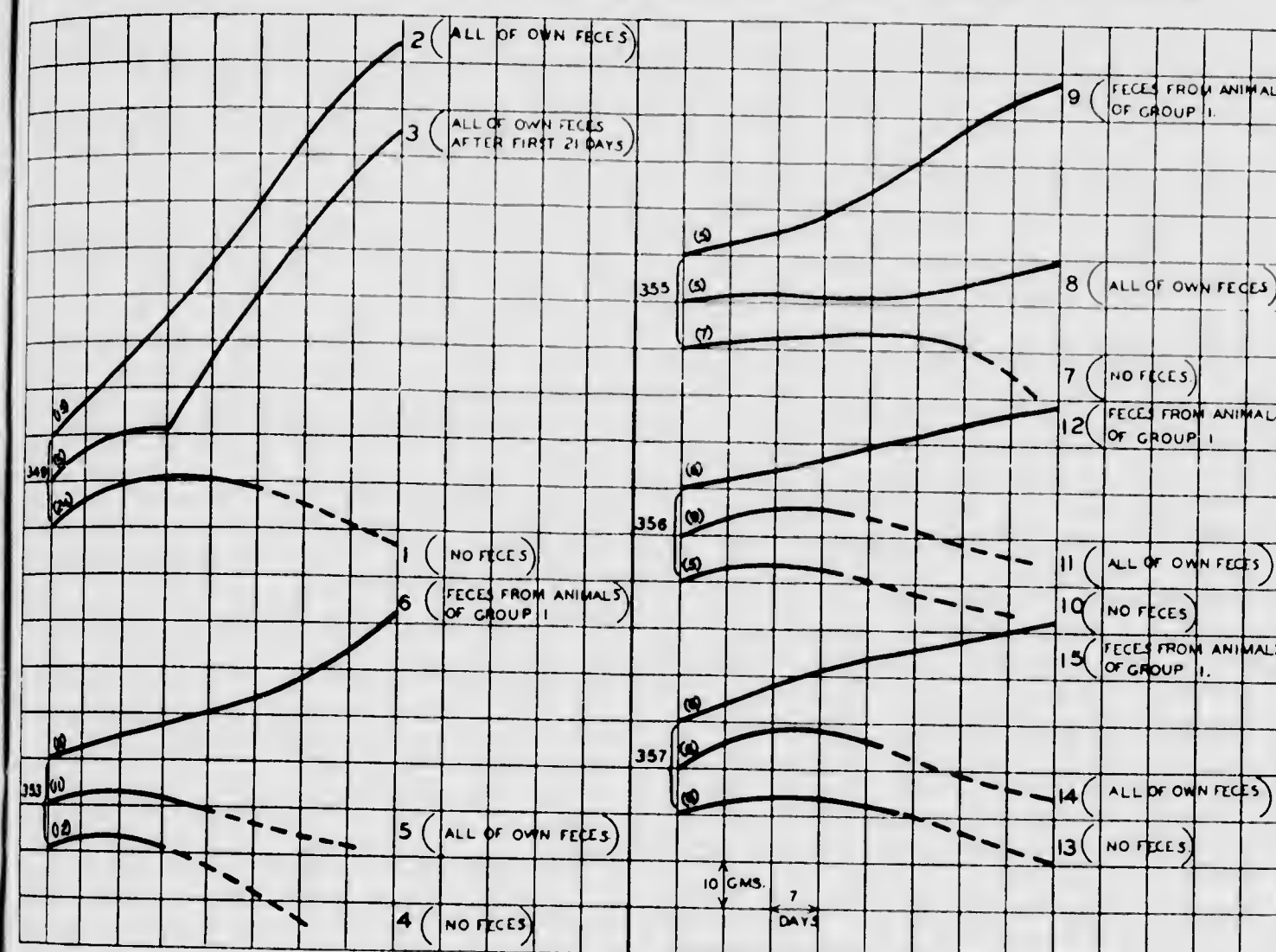


FIG. 1. Showing graphically the average growth responses made by the several groups of animals during the 8 week experimental period. The number preceding the growth curves designates the respective diets, the number in parentheses denotes the number of animals considered, and the numbers following the growth curves designate the respective animal groups. The break in the curves designates the initial death among the animals comprising the respective groups.

time was devoted in an attempt to throw some light on this subject. Any belief that the beneficial effects of the dextrinized starch was due to the retained vitamin or vitamins does not seem tenable when we are confronted with the fact that the untreated starch failed to show any such properties (Curve 14). Naturally, our thoughts turned toward an investigation of the intestinal flora of

experimental animals which were receiving some of the diets mentioned above. In our past experience with vitamin B complex-deficient diets containing dextrinized corn-starch, sucrose, and glucose as sources of carbohydrate, we have observed a very definite contrast in the nature of the feces voided by the animals given dextrin, compared to the feces voided by the animals receiving either the sucrose or the glucose diets. The feces voided by the animals given dextrin were invariably well formed, large bulky pellets, having a dark grayish color, while those voided by animals receiving the sucrose and the glucose diets were usually small black pellets of stringy or irregular form, and of such nature as to indicate a certain degree of constipation. It, therefore, seemed entirely possible that the beneficial effects of coprophagy among rats receiving diets containing dextrinized corn-starch might be the result of the action of certain microorganisms that inhabited the digestive tract of the animals when taken from the breeding colony and placed on experiment. And it also seemed that, if such was the case, the effects of coprophagy might become less pronounced as the experiment progressed. An examination of Curve 2 (Fig. 1) does not show this to be the case. In fact, when experimental animals, which received diets containing the dextrinized starch, were denied access to their feces for the first 21 days of the experimental period, and after this date were fed their own feces daily, the resultant growth responses were comparable to those produced by other animals on a similar regimen but having access to the feces from the beginning of the experiment (Curves 2 and 3). From this it appears that either the bacterial flora of the digestive tract of the rat is not readily altered by changes in the dietary habits of this animal, or else the bacterial flora in the digestive tract of the rat (when placed on experiment) is not an important consideration in this connection.

The bacteriological phase of the problem was attacked from other angles with the hope of ascertaining the etiological factor implicated. This study was conducted solely on experimental rats receiving either the dextrin- or the sucrose-containing diets, since such rats had shown the greatest discrepancies in the feces voided. The most suggestive part of this phase of the study presented itself when feces from groups of rats receiving these two diets (Diets 349 and 353) were suspended in saline solutions and streaked on

cosin-methylene agar. The results, while not definitely conclusive, were sufficiently indicative to state that cultures made from the feces of rats fed on the dextrin diet resulted in an abundant growth of non-metallic type of colonies, whereas the cultures made from the feces of the rats receiving the sucrose diet gave invariably the typical metallic colonies, and these in relatively small numbers. Such findings, however, did not prove of definite significance.

Since attempts to feed cultures of the various isolations of the non-metallic colonies or organisms to vitamin B-deficient animals as dietary supplements did not meet with success, an attempt was made to locate the particular region of this synthetic activity within the digestive tract of the rat. In order to do this, twenty-three healthy young rats were maintained on the dextrin diet (Diet 349) for 4 weeks. They were then killed and the entire digestive tract removed and cut into eight proportional lengths. The unabsorbed material was removed from each of the corresponding sections separately, dried in a partial vacuum, and fed in proportional amounts to other animals as supplements to a vitamin B complex-deficient diet. The results obtained proved unusually interesting. Growth responses were made only by those vitamin B-deficient animals which received the unabsorbed material from the cecal and the colonic sections of the digestive tract. Material which had been taken aseptically from the cecum of dextrin-fed rats and cultured on a sterile medium (made up of 95 parts of nutrient broth and 5 parts of Diet 349) for 4 days at 37° gave a profuse growth of yeast, while material taken from the cecum of the sucrose-fed rats and treated in a similar manner gave only a mixed culture with a preponderance of the colon type of organism. When the same materials were cultured on a sterile medium containing 95 parts of nutrient broth and 5 parts of Diet 353, under similar conditions, little or no growth of yeast was observed from either culture. These results indicated that this phase of the investigation was a definite problem in itself; therefore, no efforts were made to carry these particular studies beyond this point.

As a result of the above findings we were led to believe that we had finally obtained an answer to the question, why is dextrin an unsatisfactory source of carbohydrate when incorporated into diets used in assaying for the B vitamins? Since the presence of

yeast in the digestive tract had been established, little remained to be assumed as to why coprophagy among experimental rats receiving diets containing dextrinized corn-starch had vitiating effects on vitamin B and vitamin G assays. Our attention had been previously attracted by the unusually large cecum of those animals which had received the dextrinized corn-starch diet compared to the size of the cecum of other animals that had received vitamin B complex-deficient diets which contained either sucrose or glucose as the source of carbohydrate. On autopsy examination of such animals, the cecum of the dextrin-

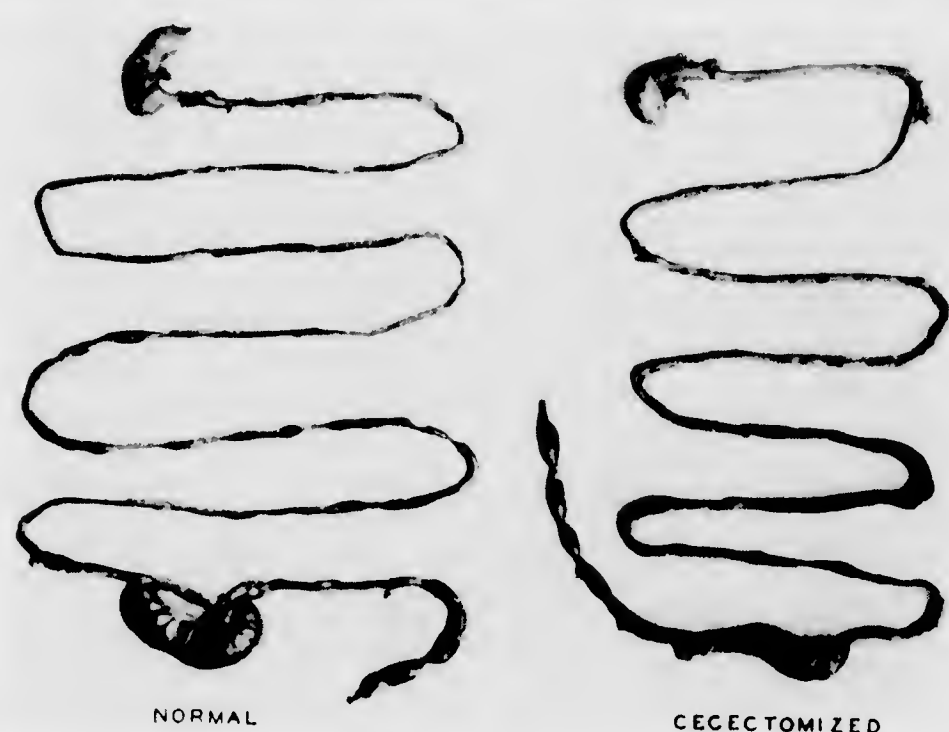


FIG. 2. Showing the digestive tract of the cecectomized rat as it appeared at the end of the experimental period, as well as that of a normal animal of comparable weight, which had been fed a diet containing dextrin as the sole source of carbohydrate.

fed rat was invariably found to be distended and filled with residual dietary material, while the cecum from the sucrose-fed rat or that of the glucose-fed rat was usually contracted and practically void of food material. Since previous tests had shown that the cecal contents of dextrin-fed rats was highly effective in supplementing a vitamin B-deficient diet, it seemed highly probable that the elaboration of the supplementary substances might take place in this organ. To determine if this was actually the case two healthy rats of about 60 days of age were anesthetized and the ceca removed (Fig. 2). Only one of the animals completely recovered from

the operation, the other having died several days after the operation, evidently as a result of intestinal obstruction. The animal that recovered was given a 6 week postoperative period, during the last 4 weeks of which it received our regular breeding colony diet. At the end of the 6 week postoperative period this animal was placed on the dextrinized corn-starch diet (Diet 349), and was maintained on this diet throughout the remainder of the experiment. During the first 28 days, the animal received this diet

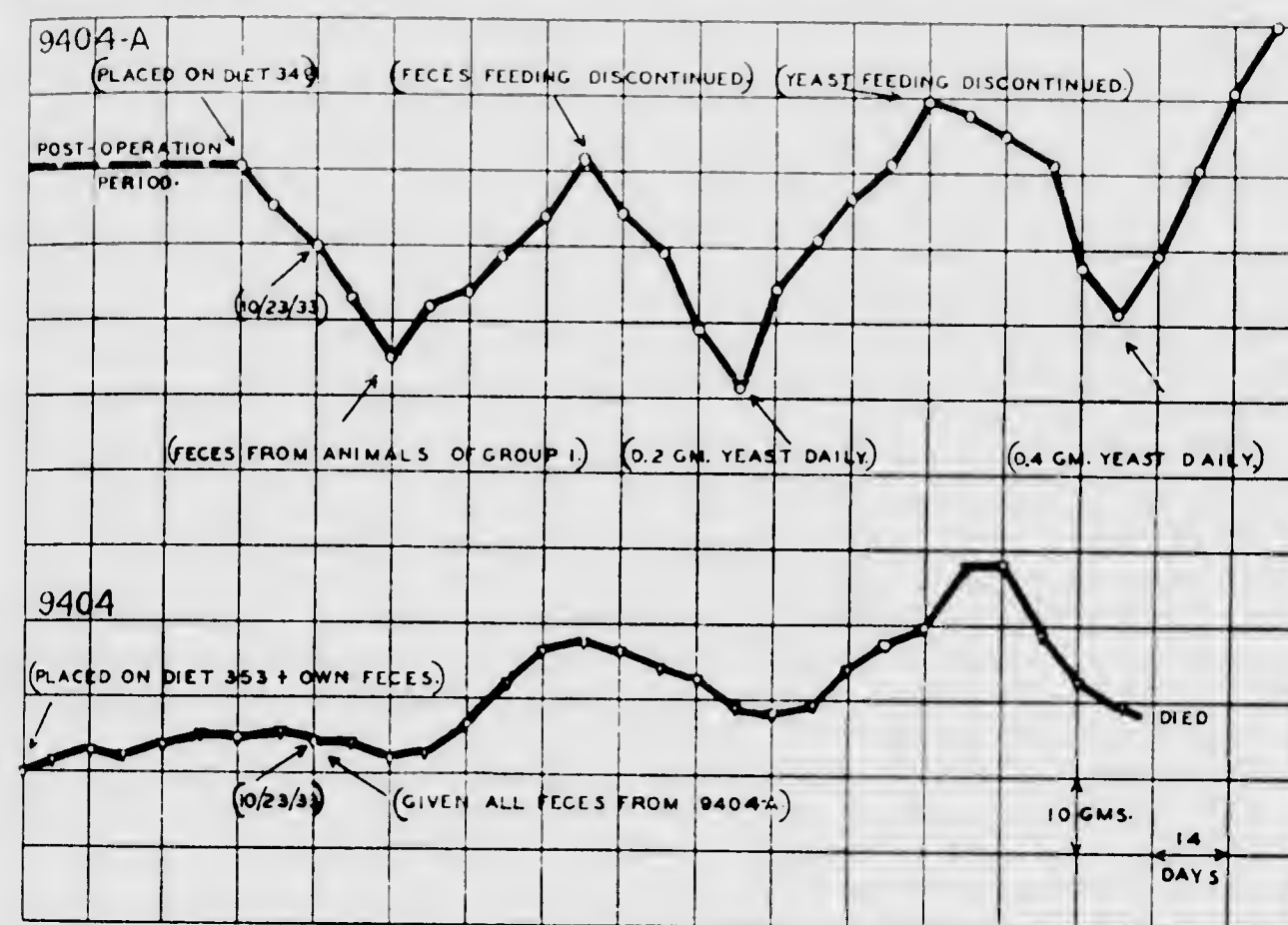


FIG. 3. Showing the responses made by a cecectomized rat (Animal 9404-A) receiving a vitamin B complex-deficient diet as a result of certain dietary supplements, as well as the responses made by another animal (No. 9404) which had access to all feces voided by the cecectomized animal.

without additional supplement, and, as the result, declined in weight to the extent of 24 gm. The animal was then fed a daily supplement of 0.4 gm. of pulverized feces which had been voided by the animals of Group 1. The feeding of feces as a dietary supplement was continued for 5 weeks, during which time the animal gained 26 gm. During the next 4 weeks the basal diet remained unsupplemented, and again the animal lost weight, this time to the extent of 31 gm. This was followed by a 5 week period, during which 0.2 gm. of dry bakers' yeast was fed daily as a dietary sup-

plement. During this yeast feeding period the body weight of the animal increased 38 gm. When this yeast feeding was discontinued, the animal declined in weight, with the resultant loss of 28 gm. during a 5 week period. But when the yeast supplement was again fed, this loss in weight was rapidly regained.

After the cecectomized animal had been on the dextrinized corn-starch diet for a 2 week period, the feces of this animal were collected daily and fed to another but younger animal which received the diet containing sucrose as the carbohydrate (Diet 358). Fig. 3 shows the various changes in regimen of these two animals and also gives the corresponding responses made as a result of such changes.

From an examination of the two growth curves given in Fig. 3, it is shown quite conclusively that Animal 9404 could not maintain growth while subsisting upon a vitamin B complex-deficient diet which was supplemented daily by the feces voided by Animal 9404-A, except when the latter animal was also receiving such dietary supplements. This would indicate that the feces from Animal 9404-A did not contain the required substances necessary to supplement a vitamin B complex-deficient diet, except when the diet of this animal was being supplemented by such dietary essentials. Such had not been the case when the animals of Group 6 had been fed the feces voided by the animals of Group 1 (Fig. 1). Therefore, it seems highly probable that the supplementary value of the feces voided by Animal 9404-A had been greatly reduced as the result of the removal of the cecum.

It is recognized that definite conclusions cannot be safely drawn from the observation on only one animal or a pair of animals. The data obtained in this connection are significant, however, in that they substantiate that which had been formerly obtained; namely, that it is in the cecum of the dextrin-fed rat that the unabsorbed food materials first become capable of supplementing vitamin B complex-deficient diets. Thus, it would appear that the cow is not unique in its ability to synthesize the B vitamins in its digestive tract (3). The cow does have the advantage over the rat in that the vitamins are synthesized higher up in the alimentary canal of the cow (rumen), thus enabling this animal to secure (on a ration deficient in the B vitamins) full benefits of the synthesized products without the necessity of reingestion of feces.

SUMMARY

1. Data are presented which show that the vitiating effect of coprophagy on the assay of the B vitamins is much more serious when the experimental diet contains dextrinized corn-starch as a source of carbohydrate than when the diet contains a similar amount of either sucrose, lactose, glucose, or commercial corn-starch. Lactose, however, appears to occupy somewhat of an intermediate position between the dextrans and sucrose in this respect.

2. This peculiar property of the dextrinized corn-starch is not due to the retained B vitamins, but rather to the formation of these vitamins in the lower part of the digestive tract of the rat, as a result of the incomplete digestion of this particular form of carbohydrate.

3. This dietary elaboration apparently takes place in the cecum of dextrin-fed rats.

4. Since live yeast cells were found to exist in the cecum of such rats in enormous numbers, these microorganisms are believed to be the specific agents of elaboration of the B vitamins.

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Influence of Electrolytes Added to Growth Medium on Electro-
phoretic Potential of *Escherichia Coli*.*

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Aqueous suspensions of *Esch. coli* picked from 24-hour growths at 37.5° C. on Bacto-nutrient agar adjusted in pH from 6.2 to 7.9 gave slightly lesser (to 4%) cataphoretic velocities, as measured in Falk capillary cells¹ than did suspensions washed one to 3 times. Accordingly, we used a suspension produced by washing the growth from the medium, centrifuging once and resuspending the bacteria in distilled water, in a study of the effect of the addition of salts to the growth medium on the electrophoretic velocity of *Esch. coli* as measured with the Falk capillary cell. As Table I will show, the addition of the salts to the growth medium had little or no effect on the cataphoretic velocity which measures the zeta potential. In a future paper (Pedlow and Lisse) it will be shown that the zeta potential of the bacteria in aqueous suspension can be changed when salts are added to the growth medium in greater concentrations.

It was also shown that no change of the electrophoretic velocity of organisms (once washed) greater than 2.5% was produced by adjusting the initial pH of the medium to values over the range 6.8 ± 1.9 , the pH after growth having a value 6.5 to 8.6, 5.7 to 8.6, and 6.1 to 8.6 for the upper half, lower half and the whole agar.

* Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station, May 16, 1935, as Technical Paper No. 689.

† Master's thesis.

¹ Falk, I. S., Jensen, L. B., and Mills, J. H., *J. Bact.*, 1928, **15**, 421.

TABLE I.

Salt added	Concentration in medium	Migration velocity‡ Av. of 60-80 Readings, sec.	% change from control
NaCl	0 (Control)	8.06	—
"	10 ⁻⁴ Molar	8.04	—0.25
"	10 ⁻³ "	8.01	—0.62
"	10 ⁻² "	7.92	—1.74
CaCl ₂	0 (Control)	9.18	—
"	10 ⁻⁴ Molar	8.63	—5.99
"	10 ⁻³ "	8.58	—6.54
"	10 ⁻² "	8.47	—7.73
AlCl ₃	0 (Control)	8.08	—
"	10 ⁻⁴ Molar	8.05	—0.37
"	10 ⁻³ "	7.99	—1.11
"	5 x 10 ⁻³ "	8.03	—0.62
Na ₂ SO ₄	0 (Control)	8.04	—
"	10 ⁻⁴ Molar	8.46	+5.22
"	10 ⁻³ "	8.46	+5.22
"	10 ⁻² "	8.44	+4.98
Na ₃ PO ₄	0 (Control)	7.98	—
"	10 ⁻⁴ Molar	8.03	+0.63
"	10 ⁻³ "	8.01	+0.38
"	10 ⁻² "	8.00	+0.25

‡ Migration velocity = Electrophoretic velocity minus cataphoretic velocity,
voltage and distance being constant.

Removal of Spray Residues From Apples



APPLYING A COVER SPRAY IN EXPERIMENT ORCHARD NO. 2,
AT STATE COLLEGE

THE PENNSYLVANIA STATE COLLEGE
SCHOOL OF AGRICULTURE AND EXPERIMENT STATION
STATE COLLEGE, PENNSYLVANIA

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Summary and Recommendations

Residues on Pennsylvania apples in 1934 exceeded the legal tolerances in many cases. For 1935, these tolerances have been set by the Federal Government at .018 grain of lead, and .01 grain of arsenic trioxide per pound of fruit. This means that growers who must spray in July or August to control codling moth or maggot will almost certainly be faced with the necessity for washing the fruit in order to market it legally.

Over 500 samples of Pennsylvania apples were subjected to various experimental washing treatments in 1934. It was found that the satisfactory removal of lead presented a greater problem than that of arsenic, and that certain conclusions based upon studies of the arsenic removal alone do not meet present requirements. Results of these tests, as reported in this bulletin, appear to justify the following recommendations:

Spray residues difficult to remove should be avoided by following the spraying recommendations given through the office of each County Agricultural Agent.

Fruit should be washed at harvest, not as taken from storage.

A home-made flotation type washer can be recommended for Pennsylvania conditions, especially for small quantities of fruit, but the more expensive under-brush machines are somewhat more efficient.

Hydrochloric acid, at 1.0 to 2.0 per cent concentration by weight (3 to 6 gals. 20° B. acid per 100 gals.), should be used to secure maximum residue removal without injury to the fruit. One minute exposure to the acid in a flotation machine should be satisfactory in most cases.

The necessary concentration of acid depends on the amount of lead to be removed, the spray mixture employed, and the variety of fruit. A wash containing 1.5 per cent acid should meet most needs in 1935.

The acid solution should be tested at frequent intervals and restored to the desired strength, and should be changed completely after each 1,000 bushels.

Sufficient quantities of rinse water (2 to 3 gallons per bushel of fruit) should be used to remove all traces of acid from the washed fruit.

The keeping quality of the fruit is not impaired by packing the apples wet, but the use of a drier facilitates grading and packing.

The addition of wetting agents or salt to the hydrochloric acid solution has given no consistent benefit in the removal of lead from apples sprayed according to Pennsylvania recommendations. The use of wetting agents may be justified in other states where lead arsenate has been combined with or followed by mineral oil sprays.

If apples are not cleaned satisfactorily with cold 2 per cent hydrochloric acid solution, it is recommended that the temperature of the acid be maintained at 100° F., through the use of electric immersion heaters, the introduction of live steam, or of steam coils.

Only when hydrochloric acid at 100° F. fails to give satisfactory residue removal is it recommended that the machinery be slowed down to give 1½ to 2 minutes exposure of the fruit to the acid solution.

Removal of Spray Residues From Apples

DONALD E. H. FREAR AND HARLAN N. WORTHLEY

THE POSSIBLE poisonous effects of lead and arsenic on the surface of fruit sprayed with lead arsenate have been discussed for many years. As early as 1895, the toxic effects of spray residues were considered, particularly lead arsenate, which was just coming into use as an insecticide.

The amount of arsenic which may be present in food materials was limited by the food and drugs act of 1906 to 0.01 of a grain of arsenic trioxide per pound. In some sections of the country it then became difficult to control infestations of the codling moth and still keep the amount of arsenic remaining on the fruit below the Federal tolerance limit. In 1926, investigations were begun in several states on the possibility of washing the fruit to remove the high residues present.

In Pennsylvania, lighter codling moth infestations have prevailed until recent years; consequently fewer spray applications have been necessary. Relatively small amounts of spray residues remained on the fruit at harvest; hence the problem of residue removal was not important in this state. About 1930, however, it became apparent that the codling moth population was increasing rapidly and that it would be necessary to increase the number of spray applications in order to produce clean fruit. The situation became acute in 1933, when the Federal authorities placed a limit on the amount of lead as well as of arsenic. In 1933, many Pennsylvania orchardists sprayed more lightly than the codling moth infestation demanded, in an attempt to meet the legal residue limits without washing. This permitted the insects to increase at an alarming rate. In 1934, a large number of growers were faced with the necessity of washing their fruit, since the number of necessary spray applications left a residue of arsenic and lead several times the legal tolerance, which then was .019 grain of lead per pound of fruit and .01 grain of arsenic per pound of fruit. The legal tolerances for 1935 have been set at .018 grain of lead per pound of fruit and .01 grain of arsenic per pound of fruit. To meet these tolerances, fruit washing will be necessary for those growers who face the necessity of spraying in July or August for the control of codling moth or apple maggot.

In order to answer some of the most pressing questions on washing methods under Pennsylvania conditions, experiments were begun at State College and in Adams County during 1934.

How Spray Residues May Be Reduced

Any method, mechanical or chemical, which will remove enough spray residue without injuring the fruit is satisfactory. Two main methods have been suggested: dry brushing or wiping, and washing.

¹ Publication authorized May 27, 1935.

Dry Brushing.—Dry brushing is used extensively in Pennsylvania for the removal of peach fuzz. Attempts have been made to use the brushing machines for removing spray residues from apples. They are quite unsatisfactory for this purpose, as is shown in Table 1. The brushes become loaded with residue; as a result, some lots of apples may carry more residue after dry brushing than before. This method cannot be recommended for the removal of spray residues.

TABLE 1. AMOUNTS OF LEAD AND ARSENIC REMOVED FROM APPLES BY DRY BRUSHING

Grain per pound	Before brushing	After brushing
Pb	.021 .009 .014	.027 .013 .008
As ₂ O ₃	.007 .004 .006	.006 .005 .003

Hand Dipping.—For small quantities of apples, hand dipping of fruit in picking crates or other slatted containers has been suggested. The apples are dipped into a tank containing the wash solution and allowed to remain there for the required time, usually from one to two minutes. They are then lifted out and rinsed thoroughly in a tank of clean water. This method is fairly satisfactory for a few hundred bushels, but the labor cost is high, and the splashing of the wash solution, which usually is corrosive, makes this method impractical for a crop much larger than 1,000 bushels.

Washers

Various washing machines have been devised. All operate on the same general principle; the apples are carried along by some mechanical means, and subjected first to the wash solution for the required length of time, and then to a rinse solution.

Flotation Washers.—These are the simplest type from the mechanical point of view. They consist of rotating paddles or conveyor slats which push the apples along as they float through the washing solution and the rinse water. Although not quite so efficient as washers having a scrubbing action, flotation washers have the advantage of low cost and may be constructed by anyone possessing some mechanical ability.

Residue removal by a flotation washer with cold acid wash solution is satisfactory when the residue is not too great and is not complicated by heavy applications of mineral oil. Table 2 gives typical results secured with a home-made flotation washer on different varieties of fruit, with two levels of acid concentration.

The residue levels found were not always in proportion to the number of cover sprays applied. This indicates variations in thoroughness of application in different orchards. The effect of adding skim milk to the



FIG. 1. SMALL, HOME-MADE FLOTATION TYPE FRUIT WASHER (SEE FIG. 2) WITH CAPACITY OF 1000 BUSHELS A DAY

spray mixture is shown in Tables 2 and 6. The larger apples retain less residue at harvest, when this is figured as grains per pound of fruit. For example, a sample of York Imperial was separated on the basis of diameter into three sizes: 2 to 2½ inches, 2½ to 3 inches, and 3 inches up. For these three sizes, the lead residues were respectively, .030, .024, and .022 grain per pound, and the arsenic trioxide .013, .012, and .010 grain

TABLE 2. TYPICAL RESULTS OF RESIDUE REMOVAL FROM APPLES, GRAIN PER POUND, 1 MINUTE EXPOSURE—FLOTATION WASHER

Sample No.	Variety	Number cover sprays	Before washing		Residue after washing in					
					0.5% HCl		1.0% HCl		2.0% HCl	
			Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃
A-1	Red Bird	4	.034	.008	.035	.001				
A-4	E. Harvest	4	.030	.011	.006	.002				
A-76	Smokehouse	4	.056	.026			.016	.007		
A-32	Delicious	6*	.018	.011	.020	.010	.018	.008	.011	.003
A-87	Stark	5	.026	.015			.011	.005		
A-24	Jonathan	3	.033	.008	.012	.005				
A-83	Jonathan	7*	.050	.025	.026	.011	.018	.009		
A-123	Grimes	6	.032	.016			.010	.002		
A-127	Grimes	6*	.067	.022			.025	.008	.015	.002
A-218	Stayman	1	.051	.016			.012	.007		
A-369	Stayman	6*	.075	.022			.018	.004	.010	.003
A-163	York	4	.025	.007			.017	.006		
A-208	York	6*	.054	.022			.016	.006	.012	.004
A-216	Rome	4	.051	.017			.014	.006		
A-211	Rome	6*	.067	.027			.020	.011	.015	.010

* Skimmed milk added.

per pound. In addition, apples on the lower branches invariably retain more residue than those in the upper parts of the tree.

A detailed description of a satisfactory home-made flotation washer, with scale drawings, is given on pages 11 to 13. The experimental results reported in Table 2 were secured with this type of washer.

Flood or Spray Washers.—In this type, the apples are pushed forward by a continuous series of paddles on an endless chain, or other similar means, and are subjected to a heavy spray or flood of washing solution, followed by rinse water. These washers were popular in the early days of residue removal, but apparently are not used much at present. They have one advantage over the flotation washer, in that the fruit is washed with a solution which circulates continuously, but the increased cost of construction more than offsets this advantage.

TABLE 3. COMPARISON OF SPRAY RESIDUE REMOVAL BY FLOTATION WASHER AND BRUSH WASHER AT ROOM TEMPERATURE

Variety	Lead Grain per pound			Arsenic (As ₂ O ₃) Grain per pound		
	Before washing	After flotation washer	After brush washer	Before washing	After flotation washer	After brush washer
1% Hydrochloric acid						
York	.014	.016	.014	.015	.004	.003
Stayman	.054	.016	.018	.022	.006	.006
	.049	.016	.015	.015	.003	.005
	.075	.018	.012	.022	.004	.004
2% Hydrochloric acid						
York	.044	.020	.016	.015	.006	.003
Stayman	.054	.012	.019	.022	.004	.003
	.049	.016	.011	.015	.004	.002
	.075	.010	.018	.022	.003	.003

Brush Washers.—These are equipped with rotary brushes of fiber, rubber, or other material, over which the apples pass in their progress through the machine, while the wash solution is forced over them. The fruit thus is subjected to a mechanical scrubbing action, as well as to a flood of wash solution. This combined mechanical and chemical action is, on the average, a more efficient means of removing residues than any of the methods previously described, as is shown in Table 3. The brush machine did not always remove more lead and arsenic than the flotation machine, but this was true in most cases. Because of their complex mechanism, brush washers are considerably more expensive than flotation washers.

Driers

Driers have been designed to remove the water adhering to the fruit after washing. There are two general types: the roller and the belt type, both of which remove water by means of absorbent cloths which are wrung out mechanically. These are fairly efficient but do not remove water from the stem and calyx ends of the fruit, so that the apples are still damp after

drying. The presence of moisture on the fruit when packed does not affect its keeping quality. The main advantage of drying is that fruit with the excess water removed is considerably easier to handle, especially in cold weather.

TABLE 4. COMPARISONS OF UNHEATED WASH SOLUTIONS CONTAINING SODIUM SILICATE AND HYDROCHLORIC ACID, GRAIN PER POUND

Sample No.	Variety	Before washing		After washing with			
				80 lbs.—100 gal. BW Silicate		HCl—1%	
		Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃
A 121	Hubbardston *	.084	.038	.074	.034	.042	.021
A 126	Grimes †	.084	.038	.046	.015	.033	.008
A 35	Jonathan *	.140	.024	.076	.020	.033	.013
A 210	Rome	.054	.024	.042	.016	.027	.013
A 243	York	.140	.083	.056	.027	.036	.025
A 245	Stayman	.071	.046	.026	.009	.012	.010

* Skimmed milk added

† Fish oil added

Wash Solutions

Alkaline Wash Solutions.—In the apple growing sections of the Pacific Northwest, where mineral oil sprays are applied frequently in summer, alkaline washes of sodium silicate or sodium carbonate have been used. These are less efficient than hydrochloric acid solutions under Pennsylvania conditions. Oil sprays may make alkaline washes necessary in Pennsylvania in the future, but under present conditions, in which the grower uses casein spreaders or fish oil, dilute hydrochloric acid is superior. Table 4 shows the amounts of lead and arsenic removed by solutions of sodium silicate as compared with hydrochloric acid wash solutions.

Acid Wash Solutions.—Dilute solutions of hydrochloric acid are the most efficient wash solutions under Pennsylvania conditions. The greater the strength of the acid, the more residue is removed (Table 2), but when concentrations above 2 per cent by weight are used there is danger of injuring the fruit. For most varieties, a solution containing 1.5 per cent by weight probably is most satisfactory.

Making and Testing Acid Solution

Commercial hydrochloric (muriatic) acid of 20° Baume strength, in carboys, is the cheapest way to buy the acid. The carboys hold 125 pounds, or approximately 12 gallons of acid, containing about 31.5 per cent by weight of the pure acid. To make a wash solution containing one per cent of the acid by weight, approximately 26 pounds of the commercial 20° Baume acid should be used per 100 gallons of water, or about 3 gallons of acid per 100 gallons of water. For a 1.5 per cent acid solution, 4.5 gallons should be used per 100 gallons of water. Table 5 shows the

number of gallons of commercial acid required to make wash solutions of various strengths.

The concentrated acid is very corrosive and should not come in contact with the skin or clothing. It is wise to have a bag of hydrated lime at hand to neutralize any spilled acid. The acid should be measured out in glass or sound porcelain utensils.

TABLE 5. DILUTION TABLE FOR ACID SOLUTIONS

Desired concentration	Gallons of commercial HCl to add to		
% Acid	50 gal. tank	100 gal. tank	200 gal. tank
0.5	0.75	1.5	3.0
1.0	1.50	3.0	6.0
1.5	2.25	4.5	9.0
2.0	3.00	6.0	12.0

Testing.—After an acid solution has been used in the washer for some time it gradually loses strength. This is due to neutralization of the acid by the spray residues, and to dilution resulting from moisture on the fruit. Some of the acid solution usually is carried over on the surface of the apples into the rinse tank, thus resulting in a further lowering of the acid content. In order to add the required amount of water and acid, the strength of the acid must be determined. The test requires a 10 cc. transfer pipette, a 10 cc. graduated pipette, two glass tumblers, and a standard alkali solution and indicator. Standard alkali solution may be obtained from any large drug store; it should contain 23 grams of sodium bicarbonate to 1000 cc. of water, with sufficient methyl orange added to give a yellow color. The pipettes may be secured from any chemical supply company.²

The following procedure is used to determine the strength of the washing solution:—

Remove a glassful of the solution from the washer, making sure that it is well mixed. Draw the solution into the 10 cc. bulb pipette by suction until the level is above the mark on the tube above the bulb. Allow the solution to run out slowly until it is even with the mark; the pipette then contains exactly 10 cc. of the solution. Allow all of this to drain into the other clean tumbler; then, with the graduated pipette, measure the alkali solution to the upper graduation (0.0 cc. mark). Add the alkali solution in the graduated pipette slowly, with stirring, to the acid solution in the tumbler, stopping when the liquid in the tumbler changes color. Note the number of cc.'s of alkali solution used. This, divided by 10, gives the percentage of acid in the wash solution.

² While the solution mentioned above may be used for testing the acid strength, it is not satisfactory in all respects, and often cannot be obtained locally. A more satisfactory standard alkali solution made with sodium hydroxide to the same strength may be purchased from Fisher Scientific Co., 711 Forbes St., Pittsburgh, Pa., or LaMotte Chemical Products Co., Baltimore, Md. Both firms sell a complete outfit for testing washing acid solutions, including pipettes, tumblers, alkali solution, and indicator.

For example, if the solution required 8 cc. of alkali solution to neutralize it, this would indicate an acid strength of 0.8 per cent hydrochloric acid by weight. To make 100 gallons of this solution up to 1.0 per cent by weight it would be necessary to add 0.2 (1.0-0.8) x 3 gallons, or approximately 0.6 gallons of concentrated acid.

Care should be taken that all glassware is clean.

When a washer of standard size is used, the acid solution should be changed after washing approximately 1,000 bushels of apples. This represents an average day's run; it usually is most convenient to drain the washer in the evening and to make up a new solution each morning.

Heating the Solution.—It has been recommended that the acid solution be heated to between 90° and 100° F. when residues are difficult to remove. Table 6 indicates that heating the wash solution to 100° F. increased the efficiency greatly. Heating is recommended where residues are present which are not satisfactorily reduced with cold acid.

TABLE 6. INCREASE IN EFFICIENCY OF SPRAY RESIDUE REMOVAL WHEN WASH SOLUTIONS ARE HEATED

Variety	Original load	After 1% acid 60° F.	After 1% acid 100° F.	After 2% acid 60° F.	After 2% acid 100° F.
Lead (grain per pound)					
York	.052	.018	.014	.016	.015
Stayman	.009	.017	.015	.016	.014
Arsenic (grain per pound)					
York	.019	.006	.003	.005	.003
Stayman	.019	.005	.005	.004	.003

Heating may be accomplished in several ways. Where electricity is available, a 5 or 10 kilowatt immersion heater has been satisfactory, although the expense of operation is rather high. Live steam from a boiler or steam coils also appears to be satisfactory. Circulation of the acid solution directly through a heater leads to rapid corrosion and is not recommended unless acid-resisting metal is available.

TABLE 7. COMPARISON OF ACID WASH SOLUTIONS WITH AND WITHOUT WETTING AGENTS, 1 MINUTE EXPOSURE, FLOTATION WASHER

Sample No.	Variety	Grain per pound							
		Before washing		2% HCl		2% HCl plus wetting agent †		2% HCl plus wetting agent ‡	
		Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃
A-367	Stayman	.049	.015	.016	.004	.009	.001	----	----
A-369	Stayman*	.075	.022	.010	.003	.016	.003	----	----
A-370	Stayman†	.082	.017	.020	.005	.016	.004	----	----
A-366	York	.044	.015	.020	.006	----	.002	----	----
A-368	York*	.054	.022	.012	.004	.011	.001	----	----
A-370	York†	.060	.020	.020	.006	.015	.004	----	----
A-74	Jonathan*	.088	.013	.023	.008	.024	.011	.022	.009
A-125	Grimes†	.084	.032	.024	.011	.024	.010	.025	.011
A-165	Stayman*	.038	.013	.008	.003	.009	.006	.008	.004

* Plus skimmed milk, 2 lbs.—100 gal.

† Plus fish oil, 2 qts.—100 gal.

‡ Vatsol, 8 lbs.—100 gal. + De Gras, 1 qt.—100 gal.

§ Areskap, 1 gal.—100 gal.

Wetting Agents.—These lower the surface tension of the wash solution and allow the waxy surface of the apple to be wet completely. Theoretically, they should allow a more complete removal of spray residues. Several of these materials were tested on Pennsylvania fruit in 1935. Representative results are given in Table 7. In general, no more residue was removed when the wetting agents were added than when acid alone was used. Wetting agents may have a place, however, in washing apples that have been sprayed heavily with mineral oil-lead arsenate sprays.

Salt.—The addition of common salt to acid wash solutions has appeared, in some other sections, to facilitate residue removal. Tests with Pennsylvania apples in 1934 (Table 8) indicated that, while salt may have increased the removal of arsenic in most instances, it interfered with the removal of lead.

TABLE 8. THE EFFECT OF ADDING SALT TO WASH SOLUTIONS OF HCl, 1 MINUTE EXPOSURE, FLOTATION WASHER

Sample No.	Variety	Grain per pound							
		Before washing		1% HCl		1% HCl plus 1% salt		1% HCl plus 10% salt	
		Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃	Pb	As ₂ O ₃
A-25	Smokehouse	.070	.017	.020	.008	.019	.007	----	----
A-35	Jonathan	.140	.024	.033	.013	.034	.013	.037	.007
A-126	Grimes	.084	.038	.033	.008	----	----	.048	.011
A-243	York	.140	.083	.036	.025	----	----	.040	.016
A-245	Stayman	.071	.046	.012	.010	----	----	.014	.036

Rinsing

Rinsing the fruit is very important in any type of washer. Fruit improperly rinsed will tend to develop injury in storage, because of the action of the acid on the lead arsenate remaining on the surface of the apples; this sets free soluble arsenic and results in arsenical injury, particularly around the calyx end of the fruit. The amount of rinse water used should be as large as is economical; at least 2 to 3 gallons of fresh water should be used for each bushel of apples washed. A satisfactory qualitative test for thoroughness in rinsing is to touch the tip of the tongue to the calyx opening of the fruit as it comes from the washer. If rinsing is not complete the sour taste of the acid is noticeable.

Factors Influencing the Effectiveness of Residue Removal

Spray Applications.—The common spray treatments in Pennsylvania appear to offer no great difficulty to the removal of the residue, if late applications in excess of the spraying recommendations are avoided. Lead arsenate-lime sulphur alone, or used with casein or skim milk spreaders, left a residue which was not difficult to remove if present in amounts less than approximately twice the tolerance (0.040 grain of lead per pound; 0.020 grain of arsenic per pound). Sprays containing fish oil left a residue more difficult to remove. Sprays containing lead arsenate and mineral oil have not been used enough in Pennsylvania to make any conclusion

possible, although experiments in other states have indicated that the residues produced by such sprays are extremely difficult to remove; hence oil sprays should be avoided so far as possible.

Variety.—The varieties of apples used in this investigation showed considerable difference in ease of washing. Stayman and Smokehouse cleaned most readily, followed by Grimes, Rome, Jonathan, York, Stark, Yellow Newtown, and Hubbardston. The order of these varieties is tentative, some of them being represented by too few samples to permit a definite statement. Hubbardston was particularly difficult to clean. Any variety of apples may be cleaned much more readily directly after picking than later. The production of wax on the surface of the apple, which occurs when fruit is held in storage, makes the removal of spray residues more difficult.

Effect on Keeping Qualities.—Washing with dilute solutions of hydrochloric acid at concentrations of 2 per cent or less apparently did not impair the keeping qualities of marketable apples, when properly rinsed. Packing the apples when wet did not affect their keeping qualities in storage. Cull apples, however, in which there were a number of cut and bruised fruits, did not keep well after washing, apparently because of the entrance of the acid into the flesh of the fruit. The solution apparently was very difficult to remove by rinsing and exerted a harmful effect later.

SMALL HOME-MADE FLOTATION WASHER

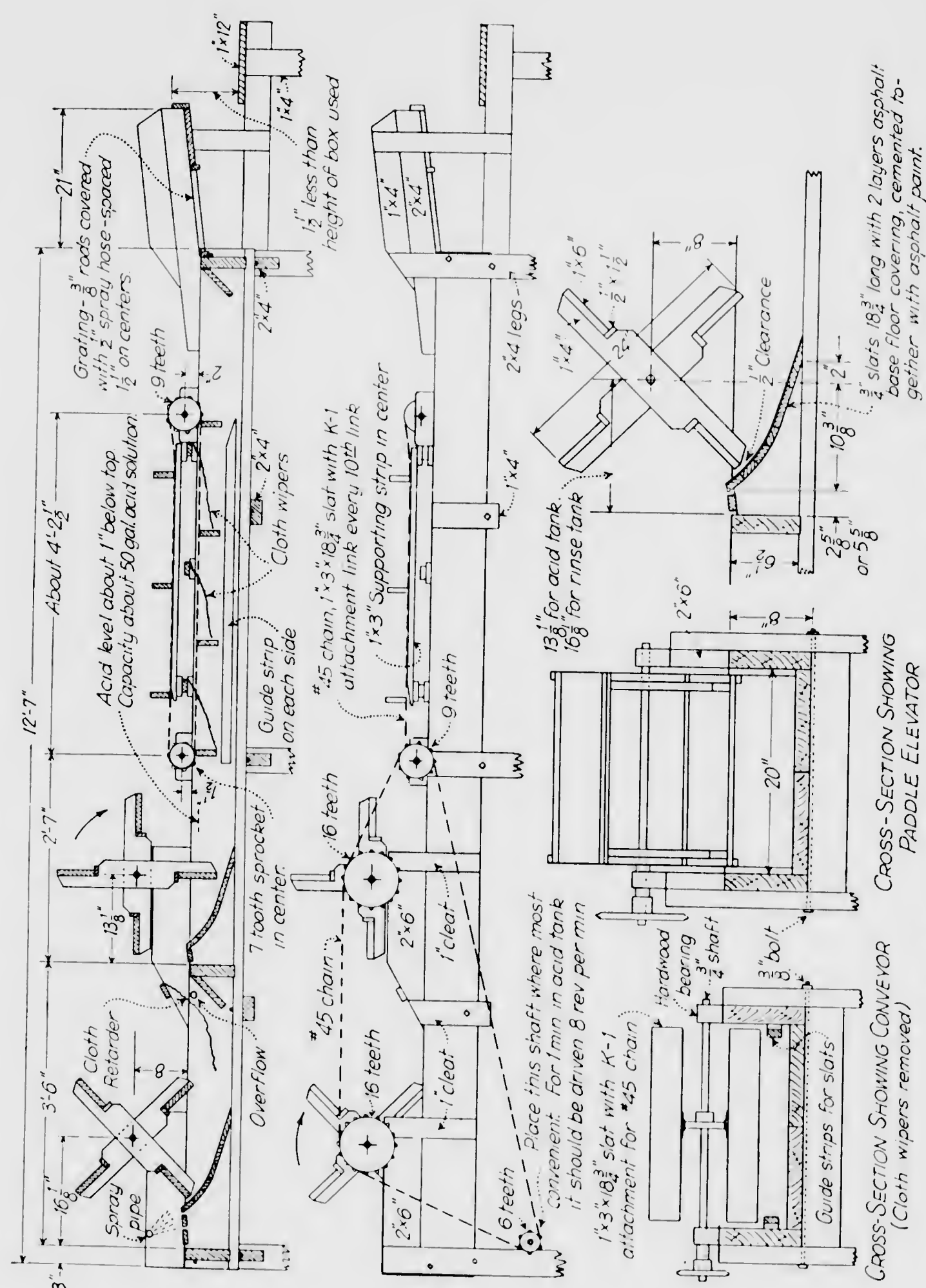
A. W. CLYDE

Fig. 2 shows a small washer in which the time in the acid tank is controlled by the speed of the machine rather than by the rate of feeding as in some home-made washers. It has a capacity of about 60 bushels per hour when apples are kept one minute in the acid tank. Apples are fed in at the right end and are carried along by the conveyor until they reach the first paddle elevator. They are then lifted out of the acid and dropped into the rinse tank. As the second paddle elevator lifts them out of the water, they are sprayed with fresh water, at least two gallons of water per bushel being advisable.

If greater capacity is desired, it will be more economical, within reasonable limits, to increase the width rather than the length. It is recommended that two chains be provided for the acid tank conveyor if the width is more than 20 inches.

Lumber one and one-half inches thick is adequate for the tank. Tongued and grooved stock is preferred. Plain edged lumber can be made watertight, however, by careful fitting of the edges and by using an asphalt roofing cement or putty. The acid solution will corrode iron or steel; hence it is good practice to countersink bolts, screws, and nails and cover them with asphalt putty. Asphalt paint should be used for the entire tank, as ordinary paints will not stand the acid.

To prevent apples being caught between the paddle elevators and the inclines, it is important that the tanks be filled to within about one inch of the top when operating. This puts the paddles about three inches deep



in the liquid at their lowest position. The cloth retarder in the rinse tank prevents bruising of apples as they drop into the tank. It is made of toweling or other heavy cloth and should be adjusted to let the apples slide gently into the tank.

The fresh water spray may be made by drilling holes in a pipe, but a few spray nozzles will be more effective.

The most practical method for getting the required speed must be worked out for each installation. As noted on the drawing, the countershaft should be driven 8 r.p.m. for 1 minute in the acid tank. With the speed and sprockets specified, the paddle elevators will run at 3 r.p.m. Apples are likely to be caught between the paddles and the inclines if the elevators run much faster than this. In many cases, power can be taken from a comparatively slow speed shaft on the apple grader in order to simplify the reduction to the slow speed required. If an electric motor is provided for the washer probably it will be best to get a worm reducing gear to give considerable of the speed reduction. The power needed is less than one-fourth horsepower. Suggestions for getting proper speed of the washer will be supplied by the Department of Agricultural Engineering, The Pennsylvania State College, State College, Pa., if information is given as to the speed of the shaft from which power will be taken.

[Reprint from the Journal of the American Chemical Society,
57, 1387 (1935).]

Methodik der Vitaminforschung. (Methodology of Vitamin Research.) By Dr. PHIL. CHRISTIAN BOMSKOV, Chemist at the University Children's Clinic of Kiel. Georg Thieme Verlag, Rossplatz 12, Leipzig C 1, Germany, 1935. xvi + 301 pp. 92 figs. 17 × 25 cm. Price, M. 24; bound, M. 26.

This volume, which contains an introduction by Professor E. Rominger, is devoted almost entirely to methods of vitamin assay and to practical and theoretical questions which bear on this important phase of vitamin research. The first 25 pages (general part) are devoted to a discussion of the types of animals used in vitamin assays with a discussion of the care and management of various types of animals used in vitamin research. He also discusses cage equipment, various types of rations used for breeding stock as advocated by representative workers, purified experimental diets, etc., accompanied by a brief discussion of the principles of feeding technique.

A second portion of the book, consisting of 132 pages, is devoted to the fat-soluble vitamins, which, for convenience of discussion, are subdivided into three main divisions, namely, Vitamins A, D and E. Each of these vitamins is discussed from the standpoint of deficiency symptoms (as well as hypervitaminosis), response of different types of animals to vitamin therapy, methods of assay (biological, chemical and physical), feeding and management technique, provitamins (such as carotene and ergosterol), isolation and purification of the fat-soluble vitamins, evidence for their chemical constitution and methods of expressing vitamin potency in units, including a discussion of International units for Vitamins A and D.

The third portion of the book, consisting of 131 pages, is devoted to a detailed discussion of the water-soluble vitamins and the organization of subject matter and method of treatment are fundamentally the same as that described for the fat-soluble vitamins. All important methods and techniques for the assay of vitamins B (B₁), G (B₂), B₃, B₄, B₅ and B₆ are treated briefly but no important details seem to have been omitted. The chemistry of the flavines and their possible relationship to Vitamin G (B₂) are also discussed. All statements and conclusions as well as all methods, experimental diets, etc., are supported by references to the original literature. Vitamin C receives very thorough treatment from practically every angle, including the most recent methods of chemical assay and the chemical synthesis of ascorbic acid. All experimental data and tabular material taken from American and English scientific journals are reproduced in English with citations to the original literature.

The reviewer feels that the book is unique in that it is the only book with which he is familiar which is devoted exclusively to this subject. "Methodik der Vitaminforschung" should prove a valuable addition to the reference shelf of every laboratory in which vitamin research is stressed.

R. ADAMS DUTCHER

Reprinted from PLANT PHYSIOLOGY, 10: 569-574, 1935.

PHOTOELECTRIC APPARATUS FOR MEASURING LEAF AREAS¹

DONALD E. H. FREAR

(WITH THREE FIGURES)

The need to measure accurately the area of several thousand apple leaves in connection with a study of the deposit of spray residues led to a search of existing methods of leaf area measurement. The several methods described in the literature failed to satisfy the requirements, inasmuch as accuracy in area measurements was apparently to be secured only by methods too lengthy to be of use in this study.

Existing methods for measuring leaf areas include the following: (1) Measurement of the leaves along their axes, the area obtained by multiplying these figures being corrected by a factor; (2) planimeter measurements; (3) tracing or otherwise transferring the leaf outline to paper of known weight per unit of area and later determining the area of the tracings by their weight; (4) leaf-punch methods, in which definite areas of the leaf surface are removed by a punch or die.

The first of these methods is not sufficiently accurate, since variations in leaf shape among individual leaves of the same species is sufficient to introduce gross errors into the calculation. The second and third methods are extremely time-consuming, depend largely for their accuracy on the skill and dexterity of the person using them, and are adapted only for measuring the areas of small numbers of leaves. Circles or other geometric figures cut from the leaf surface with a punch or die are subject to errors arising from the obvious fact that the leaf presents neither a uniform surface nor an interior structure free from gross tissue differentiation.

The use of the photoelectric cell in a variety of ways in recent years led the writer to consider the possibility of such a device for measuring leaf areas, inasmuch as the area would be a direct function of the amount of light intercepted if the leaf were placed in the path of a beam of light. While a device was being perfected, a short news article appeared describing a similar apparatus, built by WITROW, and it was learned that a paper describing a similar apparatus had been presented at the Boston meeting of the American Society of Plant Physiologists. Definite information concerning the construction of this apparatus could not be secured, however, and the present apparatus has been developed independently, and has been used satisfactorily for the past season.

¹ Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station, as Technical Paper no. 677.

Apparatus

A rectangular box 72 cm. high, 22 cm. deep, and 22 cm. wide was constructed from plyboard, with a tight fitting door at the front. The interior of the box was painted white and near the top a number of holes were bored for ventilation. A perpendicular section through the apparatus is shown in figure 1. A movable shelf *A*, to which were attached five 60-watt 110-

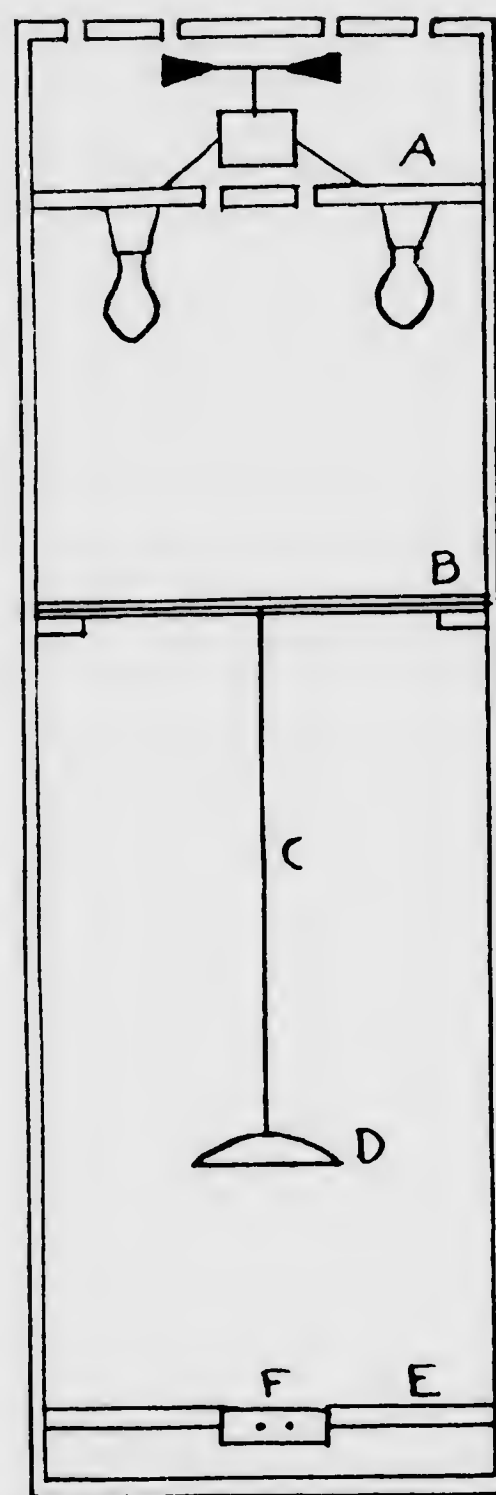


FIG. 1. Section through apparatus.

volt inside frosted electric lamps, was inserted near the top of the box. These lamps may be connected to a constant wattage transformer so that current variations may be reduced to a minimum. On this shelf was placed a small electric fan to circulate the air. Across the middle of the box was placed a pane of frosted glass, of such size that it exactly fitted the interior of the box, but masked so that only a circular opening approxi-

mately 18 cm. in diameter was available to transmit light (fig. 1 *B*). A small hole was drilled in the exact center of the circle of frosted glass so circumscribed, and a hemispherical baffle *D* was suspended by a glass rod *C*, approximately 15 cm. below the frosted glass plate. A sheet of heavy plate glass of the same size as the frosted glass was laid on top of the latter.

At the bottom of the box, about 4 cm. above the true bottom, a false bottom *E* was built, in the center of which a hole large enough to accommodate the photoelectric cell was cut. This photoelectric cell, a Weston Model 594, was connected with a microammeter having a capacity of 200 microamperes.

The purpose of the hemispherical baffle is to deflect all light rays passing through the frosted glass which would otherwise fall directly upon the surface of the photronic cell. This makes it possible for the cell to measure the *diffused* light in the lower chamber, rather than to give erratic readings because of differences in the location of shaded areas. Such differences would exist if the object causing the shading (in this case the leaves) were located in different places on the ground glass.

The intensity of the illumination in the lower chamber may be regulated by raising or lowering the bank of lamps at the top of the box.

Standardization

In standardizing the instrument, the light bulbs are connected to a 110-volt current source and adjusted so that the reading of the microammeter connected with the photronic cell is nearly the maximum of the instrument. This original reading is recorded. A section of definite area cut from a leaf similar to those to be measured is then placed dorsal side uppermost, upon the ground glass plate, and held in a flat position by the sheet of plate glass. The reading of the microammeter is again taken and the process repeated, adding a section of leaf of known area each time until the ground glass plate is covered to its capacity. A curve is then prepared, plotting the percentage of light cut off against leaf area. The percentage of light cut off is calculated by subtracting the microammeter reading taken for any given leaf area from the original reading, and dividing this by the original reading.

Variations in the electric current cause fluctuations in the reading of the microammeter, but it has been found that over the relatively short time necessary to make the standardization or to take the readings on a series of whole leaves, as will be described later, these fluctuations are not large enough to cause serious error. The use of the constant wattage transformer mentioned reduces these fluctuations greatly. It is wise to repeat the standardization several times, however, so that each point on the curve rep-

resents the mean of several readings on the microammeter. A typical standardization curve is shown in figure 2.

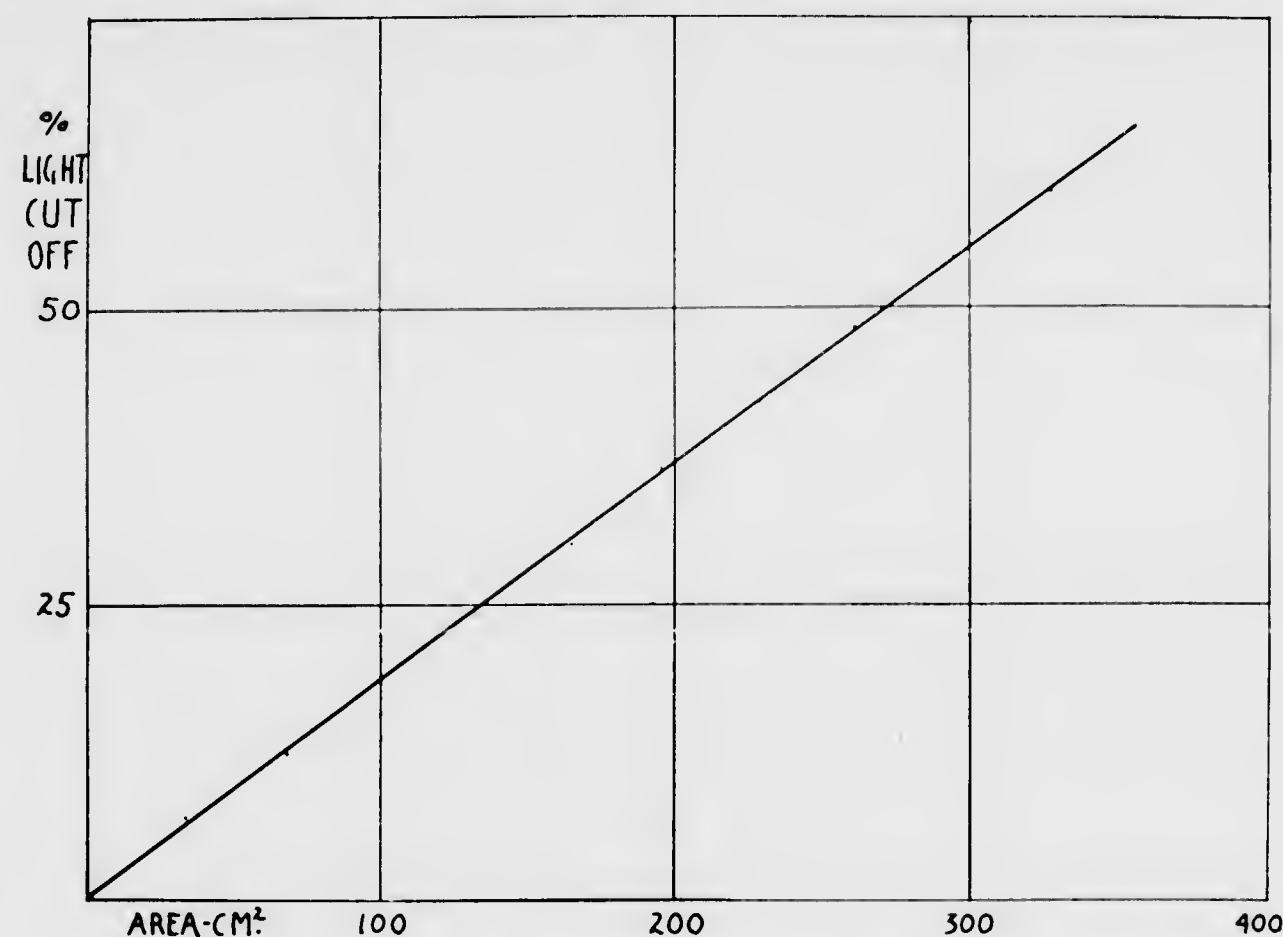


FIG. 2. Standardization curve.

Measurement of leaf areas

Having constructed a curve for the type of leaves to be measured, the actual measurement of the leaf area is carried out in a manner similar to the standardization. The original reading of the microammeter is taken without any material on the ground glass plate to interfere with the passage of the light. The leaves to be measured are then placed on the ground glass, one at a time, or, if the total area of a number of leaves is desired, several leaves at a time, and the second reading of the instrument taken. It has been found that with an apparatus of the dimensions given, four or five mature apple leaves may be placed upon the ground glass at one time, and the area of 100 leaves measured in less than 15 minutes. To guard against wide fluctuations in current, it is wise to take original readings after each five sets of leaf area readings.

The percentage of light cut off by each leaf or group of leaves is then calculated, and by reference to the standard curve, the area is determined. An example of the calculation is as follows:

Original reading	188 microamperes
Reading with 4 leaves	103 microamperes
$\frac{(188-103)}{188} = 44.0\%$ of the original light cut off.	

Referring to the curve given in figure 2, this indicates an area of 238 square centimeters.

Discussion

In building this apparatus an attempt has been made to avoid any unsymmetrical construction which might cause shadows, or tend to give a different light intensity on the photonic cell owing to a variation of the position of the leaf or leaves to be measured when placed upon the ground glass. With the instrument here described, it makes no appreciable difference whether the leaf is in the center of the circular opening on the ground glass or far to one side.

The method of standardization of the instrument may introduce serious errors in the determination of leaf areas by any photoelectric method. Leaf tissue, except perhaps in rare cases, is appreciably translucent. In addition, the reflection of light from the surface of a leaf is characteristically different from the reflection from many other materials. In fact, the reflection of light from the upper surface of a leaf is usually different from the reflection from the lower surface. This is particularly true if the lower surface is pubescent. For these reasons, then, the standardization of the instrument against known areas of opaque material, or against material of different color or reflectivity from that of the leaf surface, may lead to inaccurate results. Figure 3 shows typical curves obtained by standardizing the instrument with known areas of black paper (A); white cardboard

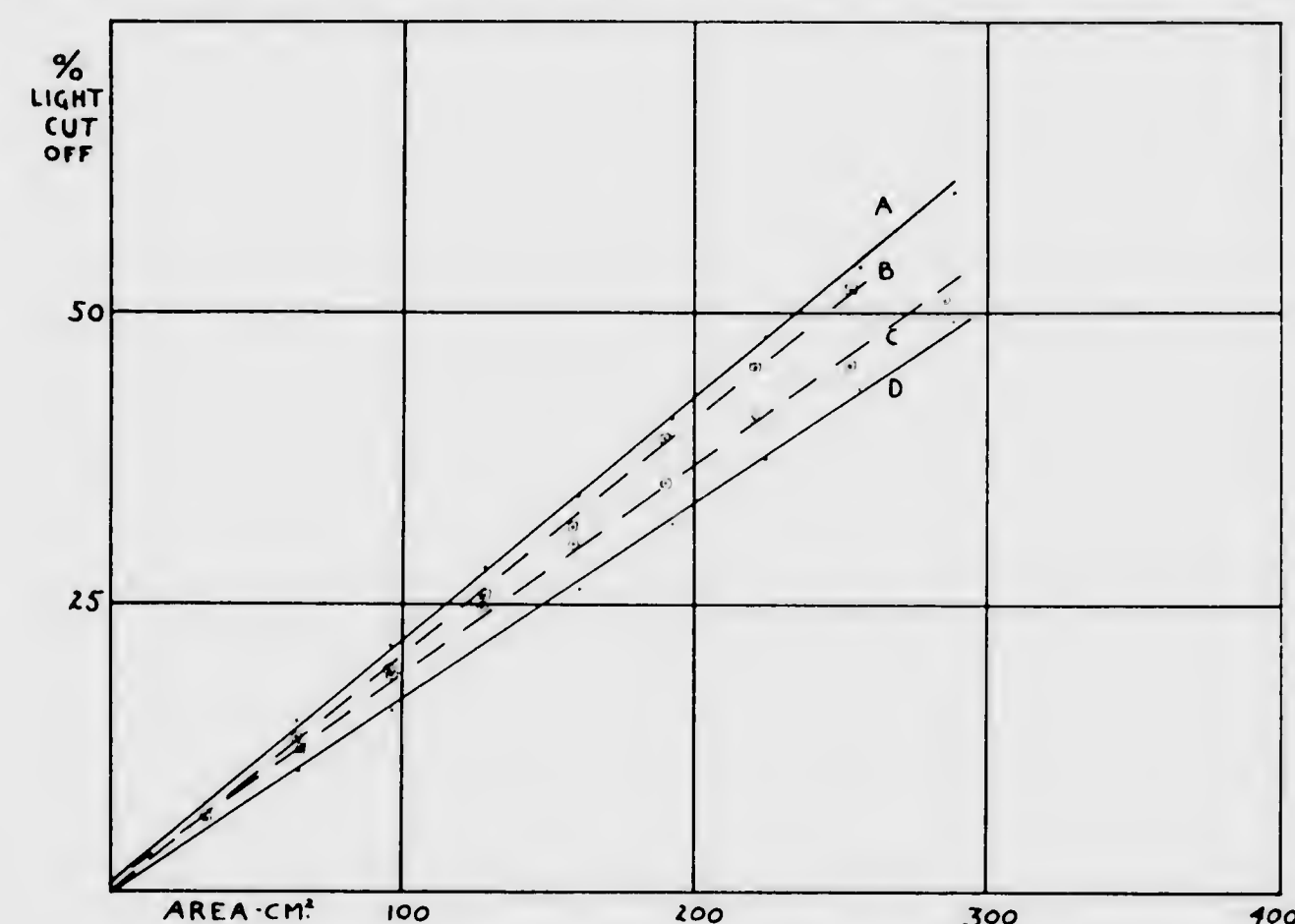


FIG. 3. Comparative standardization curves.

(D); and two types of leaf tissue, one thick and practically opaque (B) and the other relatively thin and translucent (C).

The use of alternating current to provide illumination admittedly increases the error of measurement. It is possible, however, through the use of a transformer to keep this at a minimum, and frequent checking of the current through microammeter readings helps reduce this error.

This apparatus has been used satisfactorily to measure the area of more than 10,000 apple leaves during the past season, with an error of approximately 3 per cent., as determined by replicate area measurements on the same leaves.

Summary

An apparatus is described for the measurement of leaf areas by means of the photoelectric cell. The chief advantages of the method are its rapidity and simplicity, with a high degree of accuracy. The methods of standardization and calculation are given in detail. The apparatus has been used to determine the area of more than 10,000 apple leaves, with an error of approximately 3 per cent.

DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL CHEMISTRY
PENNSYLVANIA STATE COLLEGE

STUDY OF THE REMOVAL OF SPRAY RESIDUES FROM APPLES

BY

DONALD E. H. FREAR AND H. N. WORTHLEY
(Contribution from Pennsylvania Agricultural Experiment Station)

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STUDY OF THE REMOVAL OF SPRAY RESIDUES FROM APPLES¹

By DONALD E. H. FREAR, *instructor in agricultural and biological chemistry*, and H. N. WORTHLEY, *associate professor of economic entomology, Pennsylvania Agricultural Experiment Station*²

INTRODUCTION

The problem of the removal of spray residues from apples is of comparatively recent origin in Pennsylvania, where climatic conditions and a relatively light infestation of the codling moth until 1929 combined to permit the production of clean fruit with few spray applications. Since that time, however, the codling moth populations in the commercial apple-growing centers have increased to such an extent that heavier and more frequent applications of arsenicals are now necessary. A schedule calling for five cover sprays in 1932 caused some crops to show residues of arsenic in excess of the tolerance. In 1933 restrictions were placed on lead, regarding the removal of which little was known. During the 1933 season, therefore, spraying was reduced to a minimum, with the result that excessive residues were avoided at the cost of an alarming increase in codling moth damage. It became clearly evident that intensive spraying would be necessary in 1934, and that the apple crop would doubtless require washing to meet residue tolerances.

While the fundamental facts concerning the removal of spray residues from apples are rather well understood, the fact that conditions peculiar to a locality do exist makes generalization impossible. In addition, the recent developments in methods for the accurate determination of small amounts of lead make possible a more thorough understanding of the behavior of this element under washing conditions.

During the season of 1934 a program of research in methods of apple washing was instituted, the results of which are reported in this paper.

The work was planned to give information on the method best suited for the removal of spray residues from apples grown under Pennsylvania conditions. A number of factors of climate and locality are obviously variable in any study of this kind. The extended investigations on spray-residue removal conducted at other institutions, therefore, are valuable only insofar as they can be applied to local conditions; hence some of these studies repeat, for Pennsylvania, work which has been done elsewhere. It will be noted later that certain conclusions and recommendations based on investigations in other localities do not apply here.

¹Received for publication Mar. 19, 1935; issued August 1935. Technical paper no. 682.
²The authors express their appreciation to Merrill Wood for technical assistance and to F. N. Fagan and A. W. Clyde for the use of the facilities of the Departments of Horticulture and Agricultural Engineering in this work. The authors also are indebted to S. W. Frost for the use of laboratory space, and to the fruit growers who contributed apples for experimental purposes.

EXPERIMENTAL CONDITIONS AND PROCEDURE

Part of the work was done in a laboratory set up in the largest commercial apple-growing section of Pennsylvania, in the south-central part of the State. The samples, for the most part, were collected with the cooperation of the growers of that section, but some were collected from the college orchard from experimentally sprayed trees; the work on these samples was conducted in the laboratories at State College, Pa. Over 500 samples were experimentally washed.

The varieties of apples were as follows: York Imperial, Stayman Winesap, Grimes Golden, Jonathan, Rome Beauty, Smokehouse, Delicious, Ben Davis, Stark, Hubbardston, and Yellow Newtown. The latter five varieties were represented by only a few samples.

The mean temperature for the south-central section of the State was slightly above normal during the season of 1934 except during August. The rainfall records taken at Arendtsville indicated a total precipitation for the summer months as follows: June, 6.04 inches; July, 2.51 inches; August, 5.96 inches; September, 14.09 inches. On each of 6 days during September, 1 inch or more of rain fell; the rainfall totaled 7.29 inches on 4 consecutive days, September 14 to 17, reaching nearly cloudburst proportions with 3.48 inches on September 17. The figures indicate nearly normal rainfall during June and July, when most of the codling-moth spraying was done, and a large excess in August and September, most of this coming in heavy showers during the latter month. Thus in 1934 spray applications were subjected to an average amount of weathering during the spraying period and to a great excess of rainfall between the time of the last application and the date of harvest, especially for the late varieties. It is questionable that heavy concentrated downpours of rain are as effective in removing spray residues as are more gentle rains scattered throughout the growth period of the fruit. Samples analyzed both before and after the heavy September rains showed surprisingly little reduction in spray residues from this cause. It would appear probable, therefore, that the distribution of rainfall rather than the total quantity has the greater effect on residue levels at the time of harvest.

Samples were taken by the authors, usually from the lower limbs of trees in the orchards. A sufficient number of trees were sampled to give a representative lot of apples of as nearly the minimum packed size as could be determined at the time of picking. An attempt was made to select apples of a fairly uniform size. It is well known that the smaller apples from the lower limbs of the trees usually carry the greatest amount of spray residue. When necessary, apples were taken from picking crates, but the same care was exercised in selecting for size and uniformity. Most samples were washed within 10 days after picking.

In the analysis of apple samples for lead, the method of Frear and Haley (3)³ was used, while arsenic was determined by the Gutzeit method, as described in the official methods (1, pp. 306-309). Both of these methods have been found satisfactory, although the accuracy of the lead determination is apparently greater than that of the arsenic estimation. The figures for the latter element are the mean of at least two determinations. The relative accuracy of the lead and

³ Reference is made by number (italic) to Literature Cited, p. 73.

arsenic methods, as well as the accuracy of sampling of apples, will be discussed elsewhere.

The apple washers used were of two types: One was a flotation type, similar to the washer designed by Jennings,⁴ but with structural modifications. These modifications did not affect the operation of the machine, the principle of operation of which was similar to several other types of flotation washers (2, 4). The other washer was a commercial underbrush type of the latest model, in which the washing solution is violently agitated and thrown over the apples as they advance over roller brushes. This machine was fitted with a roller drier, while the flotation machine was not equipped with a drying apparatus.

EFFECT OF VARIOUS FACTORS ON EFFICIENCY OF RESIDUE REMOVAL

The experimental work was divided into a study of the effects of the following factors on the efficiency of apple washing: Type of washer, type of washing solution, wetting agents, the use of heated solutions, spraying materials used, time of application, and variety of fruit.

EFFECT OF TYPE OF WASHER

In this study only two types of washer were available for most of the comparisons, the flotation and the underbrush type.

Table 1 gives the data secured by washing two varieties of apples sprayed with three different spray mixtures in flotation and brush machines. While individual samples showed considerable variation, a statistical analysis of the figures presented showed the underbrush washer to be significantly more efficient than the flotation washer in removing lead; the underbrush machine also was more efficient in the removal of arsenic, but the difference was not so pronounced.

The six samples identified in this and subsequent tables as 1, 2, and 3, are of both the York Imperial and Stayman Winesap varieties. They were secured in the college orchard and were treated as follows: Sample 1 received 6 cover sprays of 3 pounds of lead arsenate plus 8 quarts of lime-sulphur solution in each 100 gallons; sample 2 received 6 cover sprays of the same material, to which was added 2 pounds of skim-milk powder per 100 gallons; sample 3 received 6 cover sprays each containing 3 pounds of lead arsenate, 5 pounds of flotation sulphur and 1 quart of fish oil (cold-pressed menhaden, with less than 2 percent free fatty acids) in each 100 gallons of spray.

The underbrush washer was also more effective than the flotation washer when the hydrochloric acid solution was used with a wetting agent, as shown in table 2. In these studies the period of exposure to the acid was 1 minute in the flotation washer, and 40 seconds in the underbrush machine.

EFFECT OF DIFFERENT WASHING SOLUTIONS

Several washing solutions were tested for their efficiency in removing arsenic and lead residues from apples.

⁴JENNINGS, B. A. THE CORNELL APPLE WASHER. Cornell Mimeograph Bull. 279, 14 pp. 1934.

TABLE 1.—Comparative efficiency of lead and arsenic spray residue removal from York Imperial and Stayman Winesap apples by means of flotation and underbrush washers, various percentages of hydrochloric acid being used

YORK IMPERIAL, LEAD							
Percent- age of hydro- chloric acid in wash	Tempera- ture (° F.)	Grain of lead or arsenic per pound of fruit in—					
		Sample 1		Sample 2		Sample 3	
		Flotation- washed	Brush- washed	Flotation- washed	Brush- washed	Flotation- washed	Brush- washed
Before washing		0.044	0.044	0.054	0.054	0.060	0.060
1	60	.016	.014	.016	.018	.025	.020
2	60	.020	.016	.012	.009	.020	.021
1	100	.019	.009	.015	.010	.016	.013
2	100	.013	.016	.012	.009	.021	.021
Average		.017	.014	.014	.012	.021	.019

STAYMAN WINESAP, LEAD

Before washing		0.049	0.049	0.075	0.075	0.082	0.082
1	60	.016	.015	.018	.012	.022	.018
2	60	.016	.011	.010	.018	.020	.018
1	100	.021	.008	.020	.014	.013	.012
2	100	.012	.013	.014	.016	.016	.014
Average		.016	.012	.016	.015	.018	.016

YORK IMPERIAL, ARSENIC TRIOXIDE

Before washing		0.015	0.015	0.022	0.022	0.020	0.020
1	60	.004	.003	.006	.006	.010	.006
2	60	.006	.003	.004	.003	.006	.005
1	100	.004	.003	.006	.003	.004	.005
2	100	.003	.003	.003	.002	.005	.004
Average		.004	.003	.005	.004	.006	.005

STAYMAN WINESAP, ARSENIC TRIOXIDE

Before washing		0.015	0.015	0.022	0.022	0.017	0.017
1	60	.003	.005	.004	.004	.007	.007
2	60	.004	.002	.003	.003	.005	.005
1	100	.003	.004	.004	.006	.005	.005
2	100	.002	.001	.003	.004	.005	.004
Average		.003	.003	.004	.004	.006	.005

SODIUM SILICATE

Since sodium silicate has been used with considerable success in the apple-growing sections of the Pacific coast, an attempt was made to determine its practicability under local conditions. It was found that a cold solution of sodium silicate did not remove any appreciable amount of residue when used at a concentration of 80 pounds per 100 gallons, and even when heated to 100° F. was not so effective as hydrochloric acid. It may be mentioned at this point that few Pennsylvania growers have found it necessary to use oil sprays to any extent, and the beneficial effect of an alkaline wash is observed most readily when heavy oil applications have been made to the fruit.

TABLE 2.—Effect of wetting agents on the efficiency of lead and arsenic spray-residue removal from York Imperial and Stayman Winesap apples by means of flotation and brush washers, a 2-percent hydrochloric acid wash solution being used

FLOTATION WASHER, 1 MINUTE						
Sample	Grains per pound of fruit of—					
	Lead			Arsenic trioxide		
	Original load	After 2 percent HCl washing	After 2 percent HCl washing + wetting agent B ¹	Original load	After 2 percent HCl washing	After 2 percent HCl washing + wetting agent B ¹
York Imperial 1.....	0.044	0.020	0.015	0.006	0.002
Stayman Winesap 1.....	.049	.016	0.009	.015	.004	.001
York Imperial 2.....	.054	.012	.011	.022	.004	.001
Stayman Winesap 2.....	.075	.010	.016	.022	.003	.003
York Imperial 3.....	.060	.020	.015	.020	.006	.004
Stayman Winesap 3.....	.082	.020	.016	.017	.005	.004

UNDERBRUSH WASHER, 40 SECONDS

York Imperial 1.....	0.044	0.009	0.007	0.015	0.003	0.001
Stayman Winesap 1.....	.049	.011	.013	.015	.002	.002
York Imperial 2.....	.054	.010	.009	.022	.003	.003
Stayman Winesap 2.....	.075	.018	.018	.022	.003	.003
York Imperial 3.....	.060	.016	.008	.020	.005	.002
Stayman Winesap 3.....	.082	.018	.012	.017	.005	.003

¹ A commercial defoaming agent was added to the solutions containing the wetting agents in both types of washers. As given in this and subsequent tables, wetting agent A was Areskap, 1 gallon per 100 gallons, and wetting agent B was Vatsol, 8 pounds per 100 gallons, plus 2 quarts of Degras (Antifoam no. 16).

SODIUM CARBONATE AND SOAP

A commercial preparation composed of sodium carbonate and a coconut-oil soap was tried in various concentrations, but was not considered effective enough to warrant extended investigation.

HYDROCHLORIC ACID

Since dilute solutions of hydrochloric acid are the most widely used washing liquids, a large number of washings were made with this material. It was found that among the solutions tested it was the most effective in reducing the amount of the residues. The concentration necessary to use on various varieties showing different levels of residue was studied in some detail.

A large number of lots of apples of different varieties under various spray treatments were run through the flotation washer at room temperature, and the quantity of lead and arsenic removed by using hydrochloric acid at three concentrations was determined. The time of immersion in the washer was 1 minute; and the concentrations are given in percentage by weight of hydrochloric acid. Table 3 shows the relative effectiveness of the three concentrations in removing lead and arsenic. The results are expressed in percentage of residue (lead or arsenic trioxide) remaining on the fruit.

Maximum removal was effected by the highest concentration, and only at this concentration was the removal of arsenic proportionately as great as that of lead. This important consideration has apparently

not been mentioned in previous studies. Based on an average of all samples from various sources, the greatest efficiency of removal of both lead and arsenic was apparently at an acid concentration of approximately 2 percent by weight, when the period of immersion was 1 minute, in a flotation washer. These generalized relationships may be changed by various factors, as will be shown later.

TABLE 3.—Percentage of original arsenic and lead spray residue remaining on apples¹ after 1-minute exposure in flotation washer, various percentages of hydrochloric acid being used

Hydrochloric acid strength	Number of samples	Original residue remaining on fruit after washing	
		Lead	Arsenic trioxide
0.5.....	10	Percent 44.5	Percent 61.5
1.0.....	74	33.1	37.9
2.0.....	17	29.5	26.8

¹ See p. 62 for varieties represented in this table.

HYDROCHLORIC ACID AND SALT

The addition of salt to solutions of hydrochloric acid has been advocated by several workers (2, 7). Overley et al. (6), however, indicate questionable results at temperatures less than 110° F. An investigation of the possible usefulness of salt was undertaken. The figures in table 4 indicate that when small quantities of salt (1 percent) are added to dilute acid in a flotation washer the efficiency of the acid is not appreciably increased. When larger quantities of salt are added there is a decided decrease in the amount of lead removed, while the amount of arsenic removed is very slightly increased.

TABLE 4.—Effect of added sodium chloride on the efficiency of lead and arsenic spray-residue removal from apples with hydrochloric acid wash solution

Composition of wash solution	Number of samples	Average lead residue remaining on fruit after washing with—		Average arsenic trioxide residue remaining on fruit after washing with—	
		Acid alone	Acid + NaCl	Acid alone	Acid + NaCl
1 percent HCl containing 8 pounds NaCl per 100 gallons.....	2	Percent 26	Percent 26	Percent 50	Percent 45
1 percent HCl containing 50 pounds NaCl per 100 gallons.....	3	28	35	25	21
1 percent HCl containing 100 pounds NaCl per 100 gallons.....	3	28	35	25	21
2 percent HCl containing 100 pounds NaCl per 100 gallons.....	3	23	27	28	26

MIXED ACIDS

A mixture of 1 percent of hydrochloric acid and 0.5 percent of nitric acid was tried as a residue-removing solution. No apparent benefit was secured by the addition of nitric acid.

EFFECT OF WETTING AGENTS

The work of McLean and Weber in 1931 (5) indicated that the use of a wetting or foaming agent increased the effectiveness of washing in the removal of spray residues. Several commercial products have appeared on the market, and a test of their efficiency under Pennsylvania conditions was considered desirable. In using these various products, the recommendations of the manufacturer regarding the quantity to be used were followed, when such recommendations were given; otherwise a 1-percent solution by weight of the wetting agent was used. Table 5 gives typical results obtained. The data given in this table indicate that at least for the conditions of this test, these two wetting agents (the commercial preparations available to the apple industry) showed no consistent benefit.

TABLE 5.—Effect of 2 commercial wetting agents on the efficiency of lead and arsenic spray-residue removal from several varieties of apples, 1- and 2-percent hydrochloric acid wash solutions being used

Variety of apples	Grains per pound of fruit of—					
	Lead			Arsenic trioxide		
	Original load	After HCl washing	After HCl washing + wetting agent	Original load	After HCl washing	After HCl washing + wetting agent
Jonathan.....	0.140	0.033	0.034	0.024	0.013	0.012
Grimes Golden.....	.080	.026	.018	.023	.013	.011
Smokehouse.....	.070	.020	.018	.017	.008	.009
Hubbardston.....	.048	.028	.024	.018	.012	.009

1 PERCENT HCl AND WETTING AGENT B

Jonathan.....	0.140	0.033	² 0.029	0.024	0.013	0.017
Do.....	.098	.033	.036	.025	.013	.013
Grimes Golden.....	.083	.026	.030	.030	.011	.015
Smokehouse.....	.054	.014	.015	.028	.011	.006
Hubbardston.....	.084	.042	.043	.038	.021	.026

2 PERCENT HCl AND WETTING AGENT A

Jonathan.....	0.140	0.026	0.032	0.024	0.006	0.018
Do.....	.088	.023	.022	.043	.008	.009
Grimes Golden.....	.084	.020	.025	.038	.007	.011
Do.....	.073	.020	.017	.025	.007	.011
York Imperial.....	.122	.030	.022	.048	.009	.013
Stayman Winesap.....	.038	.008	.008	.013	.003	.004

2 PERCENT HCl AND WETTING AGENT B

Jonathan.....	0.088	0.023	0.024	0.043	0.008	0.011
Grimes Golden.....	.084	.024	.024	.032	.011	.010
Do.....	.073	.020	.024	.025	.007	.018
York Imperial.....	.124	.037	.031	.038	.008	.020
Stayman Winesap.....	.038	.008	.009	.013	.003	.006

¹ Wetting agent used with 1 percent NaCl according to manufacturer's recommendations.

² Double manufacturer's recommendations.

A further study of the effectiveness of wetting agents was conducted on apple samples dipped in a smaller amount of washing solution, closely simulating in every way the treatment secured in a flotation washer. These results are shown in table 6. The apples used in this experiment received six cover sprays each containing 3 pounds of lead arsenate, 5 pounds of flotation sulphur, and 1 quart of fish oil in each 100 gallons of spray. There was no significant increase in removal of

the residue when the wetting agents were used in addition to the acid at room temperature, and a slight but consistent apparent increase in the percentage removal when wetting agent A was used with acid solution at 100° F.

TABLE 6.—Effect of commercial wetting agents on the efficiency of lead and arsenic spray-residue removal from York Imperial and Stayman Winesap apples, when hand-dipped in 1 percent hydrochloric acid wash solution at different temperatures

Washing treatment ¹	Grain of residue per pound of fruit for—			
	York Imperial		Stayman winesap	
	Lead	Arsenic trioxide	Lead	Arsenic trioxide
Original load.....	0.095	0.038	0.076	0.025
1 percent HCl, 1 minute at 60° F.....	.024	.010	.014	.005
Plus wetting agent A.....	.018	.007	.015	.006
Plus wetting agent B.....	.024	.012	.012	.005
1 percent HCl, 2 minutes at 60° F.....	.015	.009	.012	.004
Plus wetting agent A.....	.013	.004	.009	.003
Plus wetting agent B.....	.011	.005	.011	.004
Plus wetting agent C.....	.020	.011	.012	.006
Plus wetting agent D.....	.016	.007	.024	.010
Plus wetting agent E.....	.015	.008	.020	.007
Plus wetting agent F.....		.004	.014	.004
1 percent HCl, 1 minute, at 100° F.....	.010	.005	.008	.003
Plus wetting agent A.....	.006	.001	.005	.001
Plus wetting agent B.....	.008	.003	.010	.007
Plus wetting agent C.....	.016	.006	.012	.006

¹ Wetting agents C, D, E, and F were experimental products of the Rubber Service Laboratories Co.

As a means of determining the possible effect of the type of washer on the efficiency of the wetting agent, samples of apples were washed both in the flotation washer and in the underbrush washer, a 2-percent hydrochloric acid solution and wetting agent B being used. These results have already been presented in table 2. It is apparent that there was no very decided advantage in the use of wetting agents in either washer, although the apples sprayed with fish oil (treatment 3) showed a slightly greater removal of lead when the wetting agent was used than when the acid alone was used. From all three spray residues the removal of arsenic was apparently slightly aided in some cases by the presence of the wetting agent.

On the whole, under the conditions of these tests, the use of wetting agents did not assure an increase in the amount of spray residues removed. However, some of the data showed that additional arsenic was removed when wetting agents were employed, thus indicating that in certain cases their use may be justified. The exact conditions under which wetting agents may be expected to yield benefits, however, were not revealed in this study.

EFFECT OF RAISING THE TEMPERATURE OF WASHING SOLUTION

The use of heated solutions in the washing of apples has been recommended by several investigators. Particularly when the residue present on the fruit is extremely large, the use of a warm washing solution theoretically should be one of the most economically effective methods of removal. Studies were made comparing the efficiency of hydrochloric acid wash at approximately 60° and 100° F. The complete data have been given in table 1. The percentages remain-

ing, given in table 7, indicate the average amount of residue remaining on the three samples of each variety studied.

TABLE 7.—Comparative efficiency of lead and arsenic spray-residue removal from York Imperial and Stayman Winesap apples by 1- and 2-percent hydrochloric acid wash solutions, used in flotation and underbrush washers at 60° and 100° F.

Type of washer and variety of apple	Average residue remaining on fruit after washing with—							
	1 percent HCl at 60° F.		1 percent HCl at 100° F.		2 percent HCl at 60° F.		2 percent HCl at 100° F.	
	Lead	Arsenic trioxide	Lead	Arsenic trioxide	Lead	Arsenic trioxide	Lead	Arsenic trioxide
Flotation:	Percent	Percent	Percent	Percent	Percent	Percent	Percent	Percent
York Imperial.....	36	35	33	25	33	23	29	20
Stayman Winesap.....	28	26	22	23	23	20	21	19
Underbrush:								
York Imperial.....	33	26	20	18	29	20	29	16
Stayman Winesap.....	23	31	17	28	23	19	22	16

These figures show that under the conditions of this experiment the acid solutions when heated to 100° F. were definitely more effective in removing both arsenic and lead than were the corresponding acid solutions at 60°. The effectiveness of the 1-percent hydrochloric acid solution at 100° was equal to that of the 2-percent hydrochloric acid solution at 60°. The difference in ease of removal from York Imperial and Stayman Winesap, which will be discussed later, is brought out in this table.

The data on the washings in the brush machine shown in table 7 are not as consistent as might be desired. This is in part explained by the fact that with the facilities available it was not possible to maintain a constant temperature in the underbrush washer because of the violent agitation and the consequent rapid loss of heat. In the flotation washer the temperature was constant.

EFFECT OF SPRAY MATERIALS APPLIED

The ease of removal of any given spray deposit from the surface of an apple is undoubtedly affected greatly by the factors which determine the nature of this deposit. These may be complicated by a great number of variable conditions, viz., temperature, humidity, wind velocity—inasmuch as it affects speed of drying—rainfall, the chemical composition of the spray mixture applied, and the nature of the apple surface.

It was impossible, in this study, to determine the effects of all these factors, but some attention was given to the effect that the gross composition of the spray mixture may have upon the ease of removal. The data in tables 1 and 2 show the relative ease of removal of three basic spray mixtures on two varieties of apples.

The figures in table 1 have been summarized in table 8 for the three spray treatments. The percentages in the table are true averages of the four washing treatments given to fruit of each variety receiving the three different spray mixtures.

TABLE 8.—Effect of the gross composition of the original spray used on the efficiency of arsenic and lead spray-residue removal from York Imperial and Stayman Winesap apples with hydrochloric acid wash solution used in flotation and under-brush washers

Variety and sample no.	Original residue remaining on fruit after washing					
	Lead			Arsenic trioxide		
	Flotation machine	Under-brush machine	Average	Flotation machine	Under-brush machine	Average
	Percent	Percent	Percent	Percent	Percent	Percent
York Imperial, 1.....	39	31	35	29	20	24
York Imperial, 2.....	26	21	24	22	16	19
York Imperial, 3.....	37	31	33	31	25	28
Stayman Winesap, 1.....	33	24	29	20	20	20
Stayman Winesap, 2.....	21	20	20	16	19	18
Stayman Winesap, 3.....	22	19	20	32	31	31

These data indicate that lead and arsenic behave differently under washing treatment. Both York Imperial and Stayman Winesap fruit sprayed with lead arsenate and lime-sulphur without sticker retained the least lead residue at harvest, while the inclusion of fish oil—with flotation sulphur rather than lime-sulphur—built up the greatest deposits (table 1).

Table 8 shows that the lead residue from treatment 2 which contained skim-milk powder, offered the least difficulty in cleaning. In treatment 1 (no sticker) and treatment 3 (fish oil) more of the lead was retained after washing. In treatment 3 lead was more completely removed than in treatment 1 on Stayman Winesap, but not on York Imperial.

TABLE 9.—Effect of gross composition and method of application of the original spray used on the ease of lead and arsenic spray-residue removal from Grimes Golden apples with 1-percent hydrochloric acid wash solution used in flotation washers

Spray treatment (6 sprays applied) ¹	Number of samples washed	Average residue remaining on fruit after washing	
		Lead	Arsenic trioxide
		Percent	Percent
Lead arsenate, 3 pounds, lime-sulphur, 2 gallons ¹	2	30	23
Lead arsenate, 3 pounds; ² hydrated lime, 0.5 pounds, lime-sulphur, 2 gallons.....	2	39	41
Lead arsenate, 3 pounds; flotation sulphur, 5 pounds; fish oil, 1 quart.....	3	37	0
Lead arsenate, 3 pounds; skim-milk powder, 2 pounds; lethane 410, 8.5 fluid ounces; ³ lime-sulphur, 2 gallons.....	4	37	41
Lead arsenate, 3 pounds; skim-milk powder, 2 pounds; Black Leaf 40, 1 pint; ³ lime-sulphur, 2 gallons; hydrated lime, 0.5 pounds ⁴	6	32	38
Lead arsenate, 3 pounds; lime-sulphur, 2 gallons; pine-tar soap, 1 pint.....	1	38	33

¹ All quantities given are per 100 gallons of spray.
² Containing casein.

³ In 2 sprays only, at oviposition peaks.
⁴ In last cover spray only.

With respect to residues of arsenic, table 1 shows that treatment 1 built up the least, and treatment 2, containing skim-milk powder, the greatest deposits at harvest. As in the case of lead removal (table

8), treatment 2 cleaned most readily. Treatment 3, however, apparently formed an arsenic deposit considerably more difficult to remove than treatment 1, this being more pronounced on Stayman Winesap than on York Imperial.

Further study was made on Grimes Golden apples which had received six different spray treatments. These samples were washed in the flotation washer with 1-percent hydrochloric acid solution. The results are shown in table 9. Among the spray mixtures containing added stickers or spreaders there were apparently no significant differences in the ease of removal; all showed higher percentages of lead and arsenic not removed by the washing treatment than was the case with the mixture of lime-sulphur and lead arsenate.

It should be mentioned again that the amount of the original load on the fruit undoubtedly affects the efficiency of removal; higher loads being removed to a proportionately greater extent than lower loads. In the case of the data in table 9, this circumstance makes the statement in the last paragraph more significant, since in all cases the load on the fruit sprayed with lead arsenate and lime-sulphur mixture was lower than when modifiers were added.

Tables 8 and 9 suggest that the different added materials had a preferential effect in the ease of removal of arsenic and lead from the three apple varieties represented. For instance, more arsenic than lead was removed from fruit of all three varieties sprayed with the mixture containing no added sticker. This was also true for the mixture containing fish oil on Grimes Golden and York Imperial, but not on Stayman Winesap. Lead and arsenic were removed with almost equal facility from the mixture containing skim-milk powder on York Imperial and Stayman Winesap, whereas on Grimes Golden the arsenic was retained to a slightly greater extent.

EFFECT OF SPRAYING SCHEDULE

The number of applications and date of the last application in relation to date of harvest have a direct bearing on residue levels and consequently on the washing treatment necessary to bring these residues below the tolerance. It was impossible to give separate attention to this phase of the problem. However, it will be noted that the original loads reported in tables 5 and 6 were in many cases very high and were not in all cases reduced below tolerance by the washing treatments applied. The fruit sampled came from various orchards heavily infested with codling moth. The Smokehouse and Grimes Golden received 6 cover sprays ending in mid-July, while most of the other varieties were sprayed 7 to 9 times between the petal-fall application and mid-August. The York Imperial and Stayman Winesap in tables 1 and 2 received 6 applications ending in mid-July. Here arsenic removal was satisfactory with cold 1-percent hydrochloric acid, even where fish oil was used in the spray mixture. Lead removal, except where fish oil was used, appeared to be equally satisfactory in most cases, though 1 sample of York Imperial failed to react to 2-percent hydrochloric acid at 60° F. and 2 samples of Stayman Winesap to 1-percent hydrochloric acid at 100°.

EFFECT OF VARIETY OF APPLES

In the reported work on the effect of varietal differences on the efficiency of spray-residue removal, the information is neither definite nor complete. Overley, St. John, Overholser, and Groves (6) state that the Winesaps cleaned more readily than did Esopus Spitzenburg, Delicious, and Yellow Newtown. Since the Stayman Winesap apples retained more spray residue at harvest than some other varieties when subjected to the same spray treatment, it was considered likely that this variety might offer more difficulty in cleaning. This proved not to be the case, as shown by the results given in table 10.

TABLE 10.—Effect of variety of apples on the ease of lead and arsenic spray-residue removal from the fruit with 1- and 2-percent hydrochloric acid wash solutions

Percentage of acid and variety of apple	Number of samples	Average of original remaining on fruit after washing		Percentage of acid and variety of apple	Number of samples	Average of original residue remaining on fruit after washing	
		Lead	Arsenic trioxide			Lead	Arsenic trioxide
1-percent hydrochloric acid:		Percent	Percent	2-percent hydrochloric acid:		Percent	Percent
Grimes Golden.....	20	34	32	Grimes Golden.....	6	30	19
York Imperial.....	19	36	40	York Imperial.....	5	34	35
Stayman Winesap.....	17	27	38	Jonathan.....	4	28	27
Jonathan.....	6	30	39	Delicious.....	1	23	36
Rome Beauty.....	4	32	42	Stayman Winesap.....	1	21	23
Smokehouse.....	3	28	32				
Ben Davis.....	1	30	32				
Delicious.....	1	38	57				
Yellow Newtown.....	1	63	70				
Stark.....	1	42	33				
Hubbardston.....	1	50	55				

The factor of differential spray treatments is not considered in table 10, although it probably has considerable bearing on the removal of the residues by washing. In general, however, the type of application in these samples has been the same, lead arsenate 3 pounds to 100 gallons plus skim-milk spreader. The number of applications varied, however, from 3 to 8. No particular significance may be attached to those figures representing a single sample, but they are included in this table to indicate possible relationships which may exist between variety and ease of removal.

Table 10 shows that among those varieties of which sufficient numbers were examined to make interpretation possible, Smokehouse and Stayman Winesap cleaned most readily; Grimes Golden, Rome Beauty, and Jonathan came next while York Imperial offered considerably more difficulty.

Among the varieties represented by a single sample, Ben Davis appeared to be easy to clean, Stark offered moderate difficulty, and Yellow Newtown and Hubbardston were the two hardest to clean. Hubbardston in particular seemed to be, from other experiments, the most difficult to clean of all varieties tested.

SUMMARY AND CONCLUSIONS

This study attempted to determine the effects of various factors on the removal of arsenic and lead from apples under Pennsylvania conditions.

The removal of arsenic and lead were proportional to the concentration of hydrochloric acid, but a higher concentration of acid removed proportionately greater amounts of arsenic than lead. The concentration removing both of these elements in proportional quantities was approximately 2 percent by weight.

Wetting agents were of little value in increasing the efficiency of hydrochloric acid solutions in a flotation washer at room temperature, but a slight increase in the removal of arsenic resulted from their use in an underbrush machine. This increased efficiency was too small to be of any great importance, except possibly where fish oil was used in the spray mixture.

The variety of the apples washed was an important factor in the ease of residue removal by acid solutions. Listed in the order of increasing difficulty in residue removal, the varieties studied may be arranged tentatively as follows: Smokehouse, Stayman Winesap, Ben Davis, Grimes Golden, Rome Beauty, Jonathan, Stark, York Imperial, Delicious, while the few data available indicate that Yellow Newtown and Hubbardston are two varieties most difficult to clean.

The underbrush washer was slightly more effective in removing both arsenic and lead residues than the flotation washer, even though the time required for cleaning was less in the former.

Raising the temperature of the acid bath from 60° to 100° F. increased the efficiency, a 1-percent hydrochloric acid solution at 100° being approximately as effective as a 2-percent hydrochloric acid solution at 60°. The addition of salt, as recommended by several workers, did not increase the efficiency of acid solutions. Mixed hydrochloric acid and nitric acid solutions, sodium carbonate, and soap and sodium silicate were not so effective in lowering the residue on the fruit as dilute hydrochloric acid solutions.

Apparently the type of spray mixture applied had an effect on the ease of removal of arsenic and lead; this effect was not the same for both elements and was complicated by varietal differences. Fruit sprayed with mixtures containing a skim-milk spreader cleaned slightly more readily than did those sprayed with other combinations. The addition of fish oil to the spray mixture caused a deposit of residue more difficult to remove, but not so difficult as has been suggested in the literature.

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STUDIES ON THE NICOTINE CONTENT OF CIGARETTE SMOKE

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STUDIES ON THE NICOTINE CONTENT OF CIGARETTE SMOKE¹

By C. O. JENSEN, instructor in agricultural and biological chemistry, and D. E. HALEY, professor of soil chemistry and phytochemistry, Pennsylvania Agricultural Experiment Station

INTRODUCTION

Nicotine has been praised and condemned ever since it was first isolated from tobacco by Posselt and Reimann as cited by Pictet (19a, p. 159)² in 1828. Both praise and condemnation have had little factual basis, however, for the physiological action of nicotine in tobacco smoke is still a controversial subject. Before investigators can establish the effect of inhaling nicotine in smoke, they must know how much of the alkaloid is present. Many conflicting statements are found on this subject.

A review of the literature shows that several factors may cause a fluctuation in the nicotine content of tobacco smoke, among which are the moisture content of the tobacco, the rate of smoking, the quantity left unburned, and the nicotine content of the tobacco itself.

In order to study these factors a satisfactory method for the determination of nicotine in smoke must be employed. The existing methods were therefore reviewed.

METHODS FOR DETERMINING NICOTINE IN TOBACCO AND TOBACCO SMOKE

A method for the determination of nicotine in tobacco smoke must have two particular qualifications. As in the case of tobacco itself, the method must not include the estimation of ammonia, pyridine and its derivatives, or other basic substances as nicotine. It is particularly important that ammonia be separated quantitatively from the nicotine, as shown by the data of Haley, Jensen, and Olson (8)² on cigar smoke. In fact, the ammonia content of smoke equals or exceeds the nicotine content in some cases.³ Schaarschmidt (20) has shown that pyridine is present in much smaller amounts.

A second qualification which the method must have is the ability to measure accurately small amounts of nicotine.

Picric acid reacts with nicotine to give the yellow amorphous dipicrate which gradually changes to the crystalline form. Pfyl and Schmitt (19) have used this reaction as the basis of a method for the determination of nicotine. This method with modifications was used by several German investigators (2, 6, 12, 18, 26), in the analysis of both tobacco and tobacco smoke. Pfyl and Schmitt state that the picric acid method is excellent for the determination of nicotine in tobacco smoke because ammonia, pyridine, and other bases do not interfere. But Koperina (12) states that nitrogen compounds do

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² Reference is made by number (italic) to Literature Cited, p. 274.

³ JENSEN, C. O. THE CHEMISTRY OF TOBACCO SMOKE. I. THE STUDY OF THE NICOTINE AND AMMONIA CONTENT OF CIGAR SMOKE. Thesis, M. S., Pa. State Col. 1930.

interfere in this determination. In a comparison of the Pfyl and Schmitt method (19) with Baggesgaard-Rasmussen's silicotungstic acid method (1), Heiduschka and Muth (9) showed that the two methods gave practically identical results.

At the present time Chapin's silicotungstic acid method (7) or modifications of it are widely used for nicotine determinations (3, 23, 4, 11, 16, 22, 23).

Any method in which silicotungstic acid is used as the precipitating agent solves the question of ammonia separation because ammonium silicotungstate is soluble in water (7). Pyridine is more likely to interfere in the precipitation of nicotine (7, 14, 17). However, Mach and Sindlinger (14) state that nicotine can be separated from pyridine in cold 0.5 percent hydrochloric acid solution, if the solution is made so dilute that no pyridine is precipitated. In this case only a very small amount of nicotine escapes precipitation. Baumberger (3) has stated that the nicotine content of smoke can be determined by Chapin's method without pyridine interference. Wenusch (25) used this method on cigar smoke and found that the results obtained were too high. On diluting the solutions to 10 times their original volume before precipitation, the results were the same as those obtained on the polarimeter. It was possible for Wenusch to determine nicotine by the polariscope for he used the smoke from 25 cigars for one determination.

Because of the possible erroneous results that might be obtained if too much pyridine is present, a series of determinations were made to find the concentration at which pyridine does not interfere with the precipitation of nicotine in dilute solutions.

Samples containing nicotine and added amounts of pyridine were treated as outlined by Chapin (7). One hundred cubic centimeters of a solution of nicotine and pyridine was placed in a Kjeldahl flask, made alkaline with sodium hydroxide, and steam-distilled. The solutions obtained were made up to 500 cc, and four 100-cc aliquots were taken. The first aliquot was not diluted but the other three were diluted 1-1, 1-2, and 1-3 with distilled water, acidified with HCl, before precipitation with 5 cc of 12 percent silicotungstic acid. The precipitates were filtered through weighed Gooch crucibles, dried for 3 hours at 125° C., and weighed. The results are given in table 1.

TABLE 1.—Determination of nicotine as influenced by various concentrations of pyridine

Solution analyzed	Volume of solution precipitated	Dilution	Concentration of added pyridine	Concentration of nicotine	Weight of precipitate	Nicotine calculated on basis of original sample
	cc		Percent	Percent	Grams	Percent
0.060 g nicotine in 100 cc, no pyridine.....	100	None.	0	0.012	0.1164	0.059
	100	None.	0	0.012	.1168	.059
	200	1-1	0	.006	.1141	.058
	300	1-2	0	.004	.1105	.056
	500	1-4	0	.002	.1070	.054
0.060 g nicotine and 0.20 pyridine in 100 cc.	100	None.	.04	.002	.1049	.053
	200	1-1	.02	.006	.1188	.060
	300	1-2	.013	.004	.1104	.056
	500	1-4	.008	.002	.1075	.054
	100	None.	.12	.012	.5666	.286
0.060 g nicotine and 0.060 g pyridine in 100 cc.....	200	1-1	.06	.006	.3014	.153
	300	1-2	.04	.004	.1649	.083
	500	1-4	.024	.002	.1052	.053

These results show that in solutions containing less than 0.02 percent of pyridine, there is little interference in the estimation of nicotine. However, it is also evident that as the solutions are diluted a small amount of nicotine escapes precipitation. In order, therefore, to be certain that pyridine does not interfere with the determination of nicotine, the solutions must be diluted by trial until there is no sharp drop in the weight of the precipitate.

Solutions obtained from the absorption of cigarette smoke were so treated, and there was no significant difference between the weight of the precipitates from the diluted and the undiluted samples. This agrees with the results of Baumberger (3) and Schaarschmidt and his coworkers (20). Furthermore, after 18 hours the precipitates were crystalline and settled out rapidly after stirring, which is not the case when more than 0.02 percent of pyridine is present.

Since ammonia did not interfere with the results of Chapin's silicotungstic acid method (7) and pyridine did not interfere under the conditions of the writers' tests with rather dilute smoke solutions, this method was used for the present work.

APPARATUS AND PROCEDURE

The machine shown in figure 1 was devised to smoke cigars, cigarettes or pipes with a constant length and strength of puff at the same intervals.

The cigarette *a* is held in a small calcium chloride tube *b* and the smoke is absorbed in the gas-absorption tubes *c*, *d*, *e*, and *f*. The pump *p* creates a partial vacuum in the flask *j*, and this puffs the cigarette whenever the slowly rotating valve *h* is open.

The reducing gear *g* is connected by a short piece of garden hose to the valve *h*. The reducing gear is turned by a synchronous motor *i*. In order to prevent an increase of the vacuum in *j* as measured by the water manometer *m*, air is allowed to bubble through *l* into the bottle *k* which contains oil. The amount of vacuum can be regulated by the height of the oil in *k* and also partly by the valve *n*. The calcium chloride tower *o* is used to prevent water vapor from entering the pump *p*.

Cigarettes were stored for at least 2 weeks in containers of known humidity so that the moisture content could be controlled. Five cigarettes were weighed to the nearest hundredth of a gram and smoked with a "puff" 1.6 seconds long with an interval of 6.1 seconds between puffs. The difference between atmospheric pressure and the pressure in the vacuum bottle was 15.25 inches of water, unless otherwise stated. This allowed 20 cc of air, as measured by a gas meter, to pass through the cigarette with every puff. The smoke was collected in a train of four gas-absorption tubes each containing 12.5 cc of chloroform and 12.5 cc of 0.1 N sulphuric acid.

After the smoke from the five cigarettes had been collected the chloroform-acid solution was placed in a separatory funnel together with the chloroform and water washings from the cigarette holder and the absorption tubes. The liquid was agitated and the lower chloroform layer was removed and discarded, since nicotine sulphate is insoluble in chloroform. The acid solution containing the nicotine sulphate was placed in a Kjeldahl flask and steam-distilled after the addition of 35 cc of sodium hydroxide and a few pieces of porous plate.

The volume of liquid at this point was about 300 to 400 cc. In order to keep the volume of the solution from increasing the flask was heated gently. The distillate was collected in water acidified with 15 cc of dilute HCl (1-4) until 500 cc had distilled over. After the distillation was completed the nicotine was precipitated with 5 cc of 12 percent silicotungstic acid, stirred well, and allowed to stand overnight. It was then filtered through a weighed Gooch crucible, heated in an oven at 125°C. for 3 hours, and weighed. The weight of the precipitate multiplied by the factor 0.1012 gave the weight of nicotine. This was divided by the weight of tobacco smoked to get the weight of nicotine secured from 1 g of tobacco. The data given in this paper are averages of two or more results.

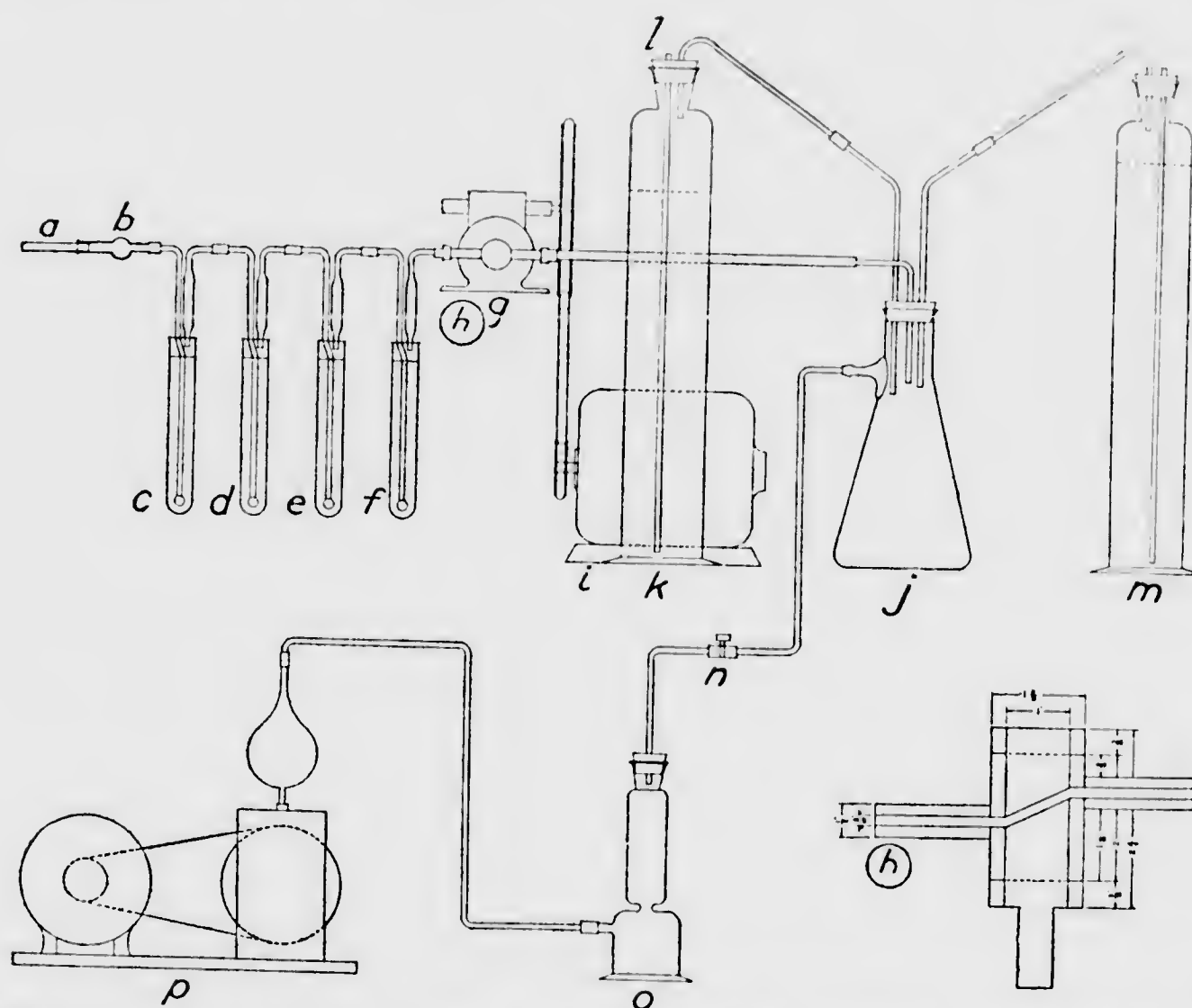


FIGURE 1.—Apparatus used to smoke cigars, cigarettes, or pipes with constant length and strength of puff at same time intervals: *a*, Cigarette; *b*, small calcium chloride tube used as cigarette holder; *c*, *f*, glass-stoppered gas-absorption tubes, 1 by 8 inches; *g*, reducing gear, 48 to 1, with 8-inch pulley; *h*, brass valve; *i*, synchronous motor, one-sixteenth horsepower, 1,200 revolutions per minute; *j*, 2-liter suction flask; *k*, tall (24-inch) bottle containing motor oil, S. A. E. 30, to a height of 17 inches; *l*, 3.5-mm glass air-inlet tube; *m*, tall (24-inch) bottle used as a water manometer; *n*, Hoke needle valve from oxygen tank; *o*, calcium chloride tower; *p*, high-vacuum pump.

The experimental conditions did not of course duplicate actual cigarette smoking conditions.

NICOTINE CONTENT OF CIGARETTE SMOKE AS AFFECTED BY THE MOISTURE CONTENT OF THE TOBACCO

In 1923 Heinz (10) stated that smoke from a moist cigar contained from 50 to 75 percent more nicotine than smoke from a dry cigar. But Schöller's results (21) in 1928 showed practically no difference between the nicotine content of the smoke of dry and moist cigars.

Winterstein and Aronson (29) published results in 1929 which differed from the findings of both of these workers. They state that from a dry cigarette 30 percent more nicotine goes into the mouth of the smoker than from a moist one. The water content of the dry tobacco was 4.8 percent and that of the moist tobacco was 16.5 percent. Waser and Stähli (24) and Molinari (15) also found more nicotine in the smoke from dry cigarettes than from moist ones. Results which differed from any yet reported were given by Kovalenko (13) in 1931, who stated that an increase in the moisture content of cigarettes from 9 to 11 percent increases the nicotine content of the smoke but a further increase in the moisture content decreases the nicotine in the smoke.

EXPERIMENTS

Cigarettes were stored for a period of at least 2 weeks in desiccators having known humidities. The desired relative humidities were obtained from the data of Wilson (27) on humidity control by means of sulphuric-acid solution.

The moisture content of the cigarettes was determined by drying in a vacuum over concentrated sulphuric acid for 2 weeks. The procedure described in the previous section was used to determine the nicotine content of the smoke from these cigarettes. The results are shown in table 2.

TABLE 2.—Nicotine content of cigarette smoke as affected by the moisture content of the tobacco

Moisture content of cigarette (percent)	Nicotine found in smoke per gram of dry tobacco	Nicotine found in the smoke as compared to nicotine in the original tobacco	Moisture content of cigarette (percent)	Nicotine found in smoke per gram of dry tobacco	Nicotine found in the smoke as compared to nicotine in the original tobacco
	Milligrams	Percent		Milligrams	Percent
0.0.....	9.2	42.4	11.10.....	5.7	26.3
3.99.....	7.8	35.9	14.34.....	5.1	23.5
6.43.....	7.3	33.6	24.44.....	4.6	21.2
9.22.....	6.5	30.0			

These results clearly show that an increase in the moisture content of cigarettes decreases the nicotine content of the smoke. Contrary to the findings of Kovalenko (13), the nicotine found in the smoke does not increase with the moisture content from 9 to 11 percent.

NICOTINE CONTENT OF CIGARETTE SMOKE AS AFFECTED BY THE STRENGTH OF PUFF

Bogen (5), Wenusch (26), and Kovalenko (13) all state that the nicotine content of cigarette smoke increases with the rate of smoking.

EXPERIMENTS

Cigarettes stored at four different humidities were smoked under conditions identical with those reported in the previous section, except that the vacuum used was equal to 14 inches of water instead of 15.25 inches. This allowed 16 cc of air to pass through the cigarette at

each puff instead of 20 cc as in the previous series. The results are given in table 3.

TABLE 3.—Nicotine content of cigarette smoke as affected by the strength of puff, cigarettes of different moisture content being used¹

Moisture content of cigarette (percent)	Nicotine found in the smoke per gram of dry tobacco		Nicotine found in the smoke as compared to nicotine in the original tobacco	
	16 cc of air puff	20 cc of air puff	16 cc of air puff	20 cc of air puff
	Milligrams	Milligrams	Percent	Percent
0.0.....	8.1	9.2	37.3	42.4
3.99.....	7.0	7.8	32.3	35.9
6.43.....	6.6	7.3	30.4	33.6
31.5.....	2.3	-----	10.6	-----

¹ In these trials 16 cc of air passed through the cigarette at each puff instead of 20 cc as in series reported in table 2.

The results show that an increase in the volume of air going through the cigarette at each puff increases the amount of nicotine in the smoke. This agrees with the findings of others (5, 13, 26).

NICOTINE CONTENT OF CIGARETTE SMOKE AS AFFECTED BY THE LENGTH OF BUTT

Heiduschka and Muth (9) determined the amount of nicotine in the smoke when four-fifths of a cigarette was smoked. Using a 4-second puff at 6-second intervals, they found an average of 0.19 percent of the weight of the cigarettes as nicotine in the smoke. The original nicotine content of the cigarettes used was 1.19 percent.

EXPERIMENTS

Cigarettes of 7-cm length with a nicotine content of 2.17 percent and a moisture content of 11.1 percent were smoked until butts of 1-, 2-, or 3-cm lengths remained. The cigarettes were given a light coating of paraffin near the end and inserted in a warm glass tube which was only slightly larger than the cigarettes. Only the part to be burned remained outside the tube. The paraffin solidified on cooling, forming an air-tight joint. The cigarettes were smoked until the burning zone reached the glass tube. The results are given in table 4.

TABLE 4.—Nicotine content of cigarette smoke as affected by the length of butt

Length of butt (length of unsmoked cigarettes, 7 cm) (centimeters)	Fraction of cigarette smoked (F)	Amount of nicotine in smoke per cigarette whose dry weight equals 1 g (A)	Nicotine found in the smoke as compared to nicotine in the original tobacco	Nicotine condensed in butt $\left(\frac{5.7 F - A}{5.7 F} \times 100\right)$
		Milligrams	Percent	Percent
3.....	$\frac{4}{5}$	1.3	6.0	60.1
2.....	$\frac{3}{4}$	2.5	11.5	38.6
1.....	$\frac{2}{3}$	3.8	17.5	22.3
0.....	$\frac{1}{5}$	5.7	26.3	0.0

The percentage of nicotine condensed in the butt was calculated from the difference between the amount of nicotine actually found and the nicotine which theoretically should have been found in the smoke $\left(\frac{5.7 F - A}{5.7 F} \times 100\right)$.

The condensation of nicotine in the unburned tobacco is a very important factor governing the amount of the alkaloid that the smoker will receive. Under the conditions of this experiment the condensation amounts to 60 percent of the nicotine which ordinarily appears in the smoke, when the length of unburned cigarette is 3 cm or three-sevenths of the original product. With a length of butt of 1 cm, only 22 percent of the nicotine which ordinarily appears in the smoke was held in the unburned portion. The actual amount of nicotine found in the smoke of 1 cigarette burned to a length of 1 cm will be approximately equal to the nicotine found in the smoke from 3 cigarettes whose butts are 3 cm in length. One cigarette smoked to a 2-cm butt will have as much nicotine in its smoke as 2 cigarettes smoked to a length of 3 cm. Although the above exact relationships will hold true only under the conditions of this experiment, it is apparent that the length of butt is one of the most important factors governing the nicotine content of cigarette smoke.

THE NICOTINE CONTENT OF THE "SIDE STREAM"

Bogen (5) states that the side stream ordinarily constitutes the greater part of the smoke as shown by the carbon-dioxide content. Winterstein and Aronson (28) measured the nicotine and reported that 43 to 62 percent of the total nicotine goes into the side stream.

EXPERIMENTS

Cigarettes were placed in the machine and smoked as usual, but instead of allowing the side stream to escape, it was trapped and passed through four gas-absorption tubes. In order to trap the side stream the burning cigarette was placed in a bulb with two small openings at opposite sides. Through one of these openings the cigarette was placed. This opening had a diameter 1 cm greater than that of the cigarette holder, which allowed air to enter the bulb at such a rate that none of the smoke was lost. The air and smoke were pulled through the other opening into the gas-absorption tubes by means of a continuously running water pump.

The results are given in table 5.

TABLE 5.—Nicotine content of the side stream smoke as modified by the moisture content of the cigarette

Moisture content of cigarette (percent)	Main stream (amount of nicotine as compared to the nicotine in tobacco)	Side stream (amount of nicotine as compared to the nicotine in tobacco)	Amount of nicotine in tobacco not found in smoke
	Percent	Per cent	Percent
11.13.....	26.3	31.8	41.9
0.0.....	42.4	28.6	29.0

Contrary to the results of the study of the relation between the moisture content of the tobacco and the amount of nicotine in the main stream, the side stream shows a decrease of nicotine when the moisture content decreases. This may be partly explained by the fact that dry cigarettes burn more quickly than moist ones. Only 8 puffs are required to burn 1 cm of the dry cigarettes, while 15 puffs are required for those with a moisture content of 11.13 percent. In the more rapidly burning dry cigarettes there is evidently greater distillation and less destruction of the nicotine. This accounts for the increased amounts of the alkaloid in the main stream. But the rate at which the tobacco burns when air is not passing through the dry cigarette may not be increased to the extent that it is when air is drawn through it. Thus the shortened time of burning will cause a smaller amount of nicotine to be found in the side stream. A study of the carbon-dioxide content of the two streams of smoke and the temperature of the burning zone might help to explain the foregoing facts.

SUMMARY

A study of methods of determining nicotine, applicable to cigarette-smoke solutions, has shown (1) that pyridine does not interfere in the precipitation of nicotine by silicotungstic acid in concentrations below 0.02 percent, and (2) that the concentration of pyridine in cigarette-smoke solutions is not high enough to interfere with the precipitation of nicotine by silicotungstic acid.

A machine is described which will smoke cigarettes, cigars, or pipes with puffs of constant volume and unvarying length at constant intervals.

The nicotine content of cigarette smoke varies inversely as the moisture content of the cigarettes.

The amount of nicotine in the smoke is directly proportional to the strength of the puff.

There is a marked condensation of nicotine in the short unburned portion of a cigarette.

Under the conditions of these experiments cigarettes with a moisture content of 11.13 percent contained more nicotine in the side stream than in the main stream; cigarettes with a moisture content of 0 contained less nicotine in the side stream than in the main stream.

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THE DISTRIBUTION AND
CONDITION OF PHOSPHORUS IN THREE HORIZONS
OF A DIFFERENTIALLY FERTILIZED HAGERSTOWN
CLAY LOAM SOIL PLANTED TO APPLE TREES
IN METAL CYLINDERS

BY

WALTER THOMAS

(Contribution from Pennsylvania State Agricultural College)

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THE DISTRIBUTION AND CONDITION OF PHOSPHORUS IN THREE HORIZONS OF A DIFFERENTIALLY FERTIL- IZED HAGERSTOWN CLAY LOAM SOIL PLANTED TO APPLE TREES IN METAL CYLINDERS¹

By WALTER THOMAS

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INTRODUCTION

The writer has recently reported (47)² the distribution and condition of the nitrogen in the soil during an experiment with apple trees grown in metal cylinders for a period of 6½ years, and which received annually for the last 3 years of growth different combinations of sodium nitrate, monocalcium phosphate, and potassium sulphate. The distribution and condition of the phosphorus is reported in the present paper. The principal objective was to determine the condition of the residual phosphoric acid (P₂O₅) derived from the added monocalcium phosphate. 98.7 and 87.3 percent of the phosphoric acid was still present in the surface 0 to 7 inches of the NPK-treated cylinders under sod and cultivation, respectively, at the end of the experiment.

The mechanism of the absorption by soils of certain anions (phosphate, tartrate, oxalate, and citrate) is comparable in certain respects to that of cations, that is, it is one of exchange involving equilibrium (8, 9, 10). The characteristics of these exchange reactions are that they take place in the boundary between two phases, and that consequently no distinction can be made between absorption, adsorption, and chemical reaction in this interface. All such reactions may be described as disperse reactions (50).

The physicochemical examination of the system phosphate, ferric hydroxide, aluminum hydroxide, calcium (and magnesium) hydroxide by Gaarder (21) has thrown considerable light on the course of the reactions that might occur under soil conditions, albeit the experiments were in vitro, i. e., they were not conducted with soils present. These experiments of Gaarder show that the systems resulting depend on the concentrations of hydrogen, iron, aluminum, calcium, and magnesium ions relative to the phosphate ions. When the conditions are such that the concentrations of iron, aluminum, and calcium are in excess of that of the phosphate only two narrow pH ranges were observed at which the concentrations of phosphate (PO₄) were more than 0.01 mg per liter, viz, at 3.9 to 4.5 and 6.7 to 7.3.

The approach to the problem of the condition of the residual phosphate applied in the writer's cylinder experiments has been made by ascertaining the total amount of phosphorus present in the three horizons, 0 to 7, 7 to 21, and 21 to 53 inches, respectively, of the soils of the treated and untreated cylinders, together with the determination of the amounts extracted from these soils under specified conditions by solvents having a wide range of pH values.

¹ Received for publication Mar. 18, 1935; issued October 1935. Pennsylvania Agricultural Experiment Station, Technical Paper no. 683.

² Reference is made by number (italic) to Literature Cited, p. 336.

METHODS OF EXPERIMENTATION

FIELD PLAN OF THE CYLINDER EXPERIMENTS

The detailed plan of this experiment has appeared in a number of publications (2, 45, 46); only a brief outline, therefore, is necessary.

The soil used in the cylinders is a virgin soil of Trenton formation formed by the weathering of limestone (44). In the present paper this soil is designated the "original soil." The excavation was made on a strip of land 110 feet by 11 feet near the college experimental orchard. The surface horizon is a heavy silt loam and is underlain by a clay loam which becomes heavier in texture with depth (44). During the excavation the three horizons, viz, surface (0 to 7 inches), subsurface (7 to 21 inches), and subsoil (21 to 53 inches), were kept separate and each pile was thoroughly mixed. The procedure of filling the boiler-plate cylinders, which were 5 feet in diameter and 5½ feet deep, has been previously described (47).

The trees were planted in the spring of 1922. The culture system, consisting of green manuring with buckwheat and rye principally, was uniform in all the cylinders until the spring of 1924, at which time half the cylinders were seeded with a mixture of Kentucky bluegrass and timothy. These cylinders are designated "cylinders under sod." The remaining half of the cylinders were kept under a system of clean cultivation. These are designated "cylinders under cultivation."

A distinction must be noted with respect to the additions of phosphoric acid from 1925 until the end of the experiment in 1927. During these last 3 years of the experiment the cylinders under cultivation received 2.5 g more phosphoric acid than the cylinders under sod. The reason for this is that it was then considered necessary to add equal amounts of organic matter to all the cylinders under cultivation. This was accomplished by growing rye outside the cylinders. For further details the paper by Anthony and Clarke (2, p. 251) should be consulted. All trees were allowed to grow without the addition of any mineral fertilizer until the spring of 1925, at which time differential treatment with different combinations of sodium nitrate, monocalcium phosphate, and potassium sulphate was commenced.

The schedule of applications of monocalcium phosphate is given in table 1.

The total amount of phosphorus (as P_2O_5) added during the experiment was 1,052.8 g. In addition to the phosphorus carried in the monocalcium phosphate the cover crops contributed approximately 10 g of phosphoric acid. The fertilizer was broadcasted and not mixed with the soil.

TABLE 1.—Schedule of monocalcium phosphate applications with phosphorus pentoxide equivalent

Date applied	CaH ₄ (PO ₄) ₂ ·H ₂ O	P ₂ O ₅ equiv- alent
	Grams	Grams
Apr. 18, 1925.....	534	300.8
May 3, 1926.....	267	150.4
June 7, 1926.....	267	150.4
May 5, 1927.....	534	300.8
May 18, 1927.....	267	150.4
Total.....	1,869	1,052.8

The total precipitation during the period of the experiment was 94.4 inches and, in addition, 2 inches of artificial watering was applied in May 1926, and 1 inch in August 1927.

Samples of the soil of each of the horizons were taken both before and after the completion of the experiment in the manner previously described (47). During the period from September 20 to 28, 1927, the trees were dug up and soil samples representative of the three horizons were taken, by the method of successive quartering, from each of the cylinders from which the trees had been removed. These samples were dried at 75° C. and then sieved through a 1-mm sieve (3) and stored in glass jars in the dark. Analyses of the trees have already been reported (46).

PERCOLATION EXPERIMENTS

CHOICE OF SOLVENTS

The solvent powers of a particular acid, barring reverse or secondary reactions, are related not only to the extent of dissociation of the salts formed and to the dissociation constant of the acid but also to the extent of hydrolysis and to complex ion formation. Moreover, it must be borne in mind that the determination by the aid of weak acids of so-called "available" phosphoric acid (P_2O_5) is not one of dissolution pure and simple but is a mechanism of exchange involving equilibrium (8, 11).

With respect to the choice of solvents one might, as some investigators have done (4, 27, 28), use only one acid, e. g., hydrochloric acid of different concentrations, thus providing a wide range of pH values. It was decided to use different acids in the present investigation not only because the different solvents chosen provide a wide range of pH values (0.7 to 5.5), but also because they have long been in use by other investigators, and thus permit a comparison of the results obtained with those of others. That fundamental differences with respect to their ability to mobilize phosphoric acid exist is indicated by the difference in their critical concentrations (10).

The choice of an acid does not appear to be altogether an arbitrary one for all types of soils, as assumed by some investigators. Laterite soils which are very high in iron oxides are decomposed by hydroxy acids with the formation of acetone and carbon dioxide (7), and some hydroxy acids, e. g., citric acid, apparently fail with certain types of calcareous soils (6), possibly as a result of a too great reduction of the acidity by the calcium carbonate.

The majority of chemical determinations of the availability of phosphate have been carried out by the "equilibrium" method. The solvents used have included principally (1) citric acid, used by Dyer (13); (2) the 0.2 normal nitric acid solvent used extensively by Fraps and his coworkers (16, 17); (3) 0.1 or 0.2 normal acetic acid solution, used in France and Germany; and (4) the solvent recently proposed by Truog (48), viz, a 0.002 normal sulphuric acid solution buffered with ammonium sulphate. Distilled water has been favored by Schloesing (40) and by Wrangell (52, 53, 54).

EXTRACTION PROCEDURE

Extraction methods in which the soil is shaken for a definite length of time with the solvent, followed by filtration, washing, and repetition of the procedure with the soil residue, are inconvenient and laborious. Percolation methods which give the same kind of results as the foregoing procedure are much more convenient. In one form or another this method has been used by many investigators. Recently Harper (25) has described a simple form of apparatus which has been further modified by the writer, as follows:

A carbon tube is fitted with a platinum cone, above which rests a layer of filter-paper pulp, followed by a layer of 40-mesh leached

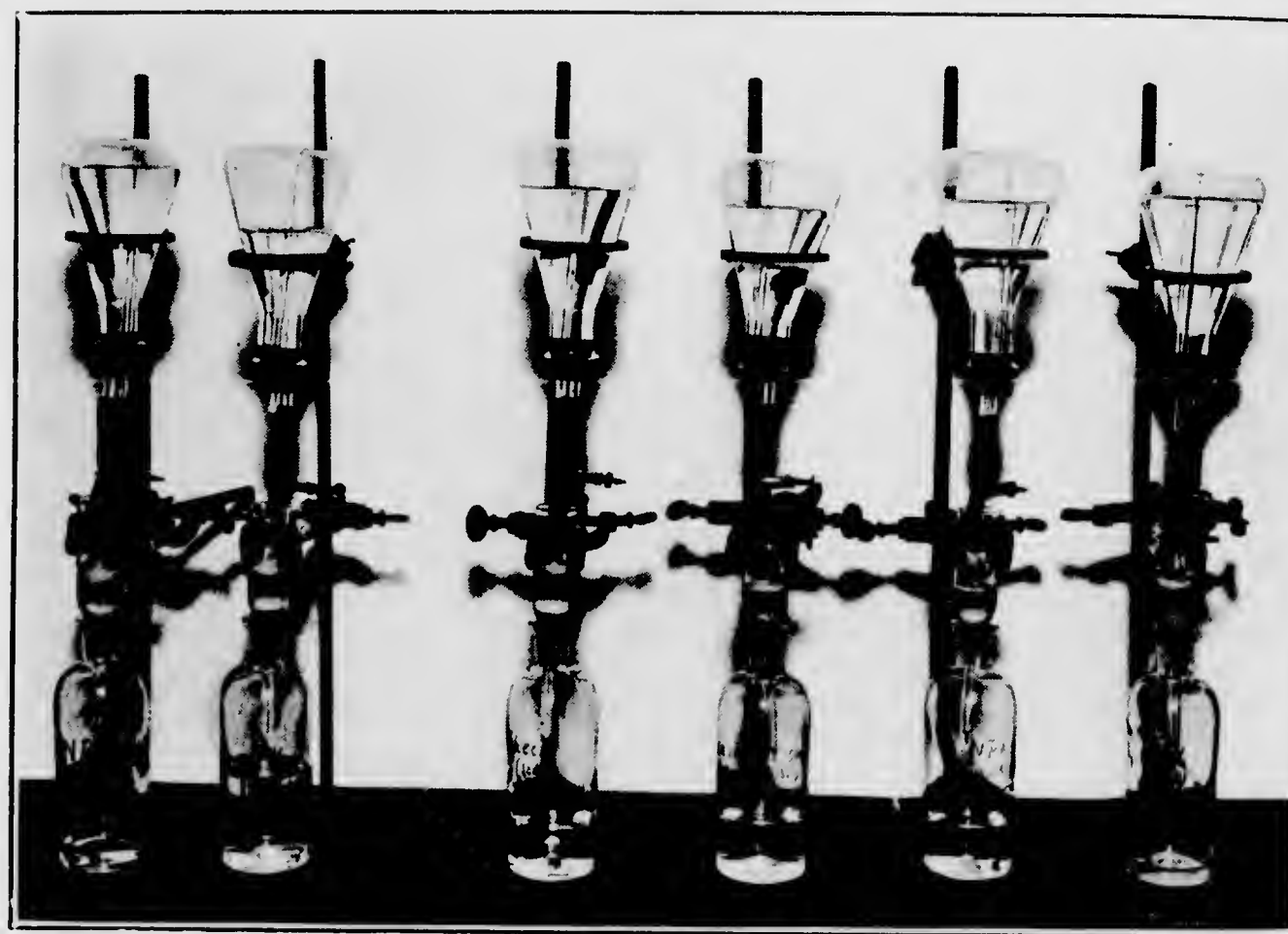


FIGURE 1.—Battery of the type of percolation apparatus used in the extraction procedure.

quartz sand. Five grams of the soil (moisture-free basis) are then added, gently compacted by tapping, and a layer of 60-mesh acid-washed quartz sand is added. The reservoir consists of a 250-cc flask fitted with air inlet and outlet tube to which is attached a piece of heavy walled rubber tubing fitted with a screw cock. This cock should work freely and be well greased. A battery of such percolation apparatus (fig. 1) is very compact and utilizes little laboratory space. The principle of the method used is one of solution and displacement, and the mechanism is similar to that described by Parker (34). The action of a dilute acid on a soil is not constant (38), and for this reason the rate of flow can be regulated sufficiently uniformly to give agreement between duplicates by making slight adjustments of the screw cock morning and evening. The extractions were made at laboratory temperature, 75° to 85° F.

DETERMINATION OF PHOSPHORIC ACID IN THE EXTRACTS

The four dilute acids used were 0.1 normal acetic (pH 2.8), 0.2 normal nitric (pH 0.7), 0.173 normal citric (pH 2.2), and 0.002 normal sulphuric buffered with ammonium sulphate (pH 3.0). Distilled water (pH 5.5) not freed from carbon dioxide was also used.

The determination of phosphorus in the distilled water and also in the acetic and sulphuric acid leachates was made by the Denigès method (12), and in the citric acid and nitric acid leachates by Richards and Godden's modification (37) of the Pemberton-Neumann method (33, 35). In the latter procedure soluble silica was previously removed by dehydration.

The blue color of the Denigès method is the result of a partial reduction of some of the phosphomolybdate. Within certain limits the color is proportional to the concentration of phosphorus in the solution. The method is very sensitive; 0.001 mg phosphoric acid in 100 cc solution can easily be detected and estimated. The literature relating to the method has been thoroughly reviewed by Zinzadze (55), who has developed a technic whereby the blue color is stable over a long period.

EXPERIMENTAL DATA

The quantities in parts per million and absolute (total) amounts of phosphoric acid in the different soil horizons are given in table 2. The results are the mean of duplicate determinations of total phosphoric acid (3), which differed by less than 0.005 percent. The results of the percolation experiments with water, sulphuric, acetic, citric, and nitric acids are given in table 3. The volume of each leachate in all cases was 200 cc.

TABLE 2.—Parts per million and absolute amounts of total phosphoric acid in the respective horizons before the trees were planted and at the end of the experiment

Treatment	Total phosphoric acid			Absolute amount total phosphoric acid			
	Surface (0-7 inches)	Subsurface (7-21 inches)	Subsoil (21-53 inches)	Surface (0-7 inches)	Subsurface (7-21 inches)	Subsoil (21-53 inches)	Total (0-53 inches)
	P. p. m.	P. p. m.	P. p. m.	Grams	Grams	Grams	Grams
Soil before trees were planted.....	1,020	950	1,020	597	1,120	2,744	4,461
Soil:							
Check.....	1,017	948	1,016	595	1,118	2,733	4,494
NPK.....	2,796	939	1,017	1,636	1,107	2,736	5,473
PK.....	2,804	948	1,018	1,641	1,118	2,738	5,476
P.....	2,819	954	1,018	1,650	1,125	2,738	5,591
Cultivation:							
Check.....	1,028	955	1,013	602	1,127	2,727	4,456
NPK.....	2,587	1,045	1,015	1,514	1,232	2,732	5,478
PK.....	2,623	1,032	1,018	1,535	1,217	2,738	5,490
P.....	2,645	1,030	1,018	1,548	1,215	2,738	5,501

TABLE 3.—Phosphoric acid in successive leachings with distilled water, and with sulphuric, acetic, citric, and nitric acids, expressed in parts per million and in absolute amounts

DISTILLED WATER (pH 5.5)

Description of soil	Phosphoric acid in successive leaching indicated															Total P_2O_5 in all leachings	Absolute amounts
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Soil before trees were planted:	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i>	<i>P. p. m.</i> ¹	Grams
0-7 inches.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
7-21 inches.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.7	5.0
21-53 inches.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
Check (sod):	8.7	(?)														5.3	6.2
0-7 inches.....	5.3	0														2.5	6.7
7-21 inches.....	2.5	0														10.2	6.0
Check (cultivation):	10.2	0														5.3	6.0
0-7 inches.....	5.3	0														2.5	6.7
7-21 inches.....	2.5	0														644.1	376.9
21-53 inches.....	229.0	109.9	77.8	48.1	37.8	28.6	22.9	18.3	17.2	13.7	10.3	9.2	8.9	6.9	5.5	3.9	6.9
NPK (sod):	5.9	0														2.7	7.3
0-7 inches.....	2.7	0														521.2	304.9
7-21 inches.....	194.5	87.5	56.2	40.3	33.7	22.3	17.4	13.8	11.9	9.8	8.7	7.6	6.5	5.9	5.1	6.8	8.0
21-53 inches.....	6.8	0														2.7	7.3

¹ Expressed in terms of the dry weight of soil.

² Trace.

0.002 N SULPHURIC ACID BUFFERED WITH AMMONIUM SULPHATE (pH 3.0)

Description of soil	Phosphoric acid in successive leaching indicated										Total P ₂ O ₅ in all leach- ings	Absolute amounts
	1	2	3	4	5	6	7	8	9	10		
Soil before trees were planted:	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	P. p. m.	Grams
	0-7 inches.....	0										0
	7-21 inches.....	0										0
	21-53 inches.....	0										0
	Check (sod):											
	0-7 inches.....	13.3	7.1	2.7	0							23.1
	7-21 inches.....	8.7	4.6	3.2	0							16.5
	21-53 inches.....	3.0	0									3.0
	Check (cultivation):											
	0-7 inches.....	17.8	12.5	7.2	0							37.5
7-21 inches.....	8.9	4.2	0								13.1	
21-53 inches.....	3.0	0									3.0	
NPK (sod):												
0-7 inches.....	230.8	343.5	230.8	76.0	34.1	6.9	6.4	2.3	(?)	0	930.8	544.6
7-21 inches.....	9.4	4.1	0								13.5	15.8
21-53 inches.....	6.4	0									6.4	17.2
NPK (cultivation):												
0-7 inches.....	234.0	278.2	251.0	109.2	41.2	8.0	6.4	2.2	(?)		913.2	534.3
7-21 inches.....	11.4	9.4	5.7	5.1	0						31.6	37.3
21-53 inches.....	6.4	0									6.4	17.2

¹ Expressed in terms of the dry weight of soil.

² Trace.

TABLE 3.—Phosphoric acid in successive leachings with distilled water, and with sulphuric, acetic, citric, and nitric acids, expressed in parts per million and in absolute amounts—Continued

Description of soil		0.1 N ACETIC ACID (pH 2.8)																			Total P ₂ O ₅ in all leachings	Absolute amounts in all leachings	
		Phosphoric acid in successive leaching indicated																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19			20
Soil before trees were planted:		P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	P, p, m.	Grams
0-7 inches		11.9	4.4	1.1	0																		10.2
7-21 inches		3.4	1.1	(3)	0																		5.3
21-53 inches		3.2	1.1	(3)	0																		11.6
Check (sod):		15.6	6.6	1.1	0																		13.6
0-7 inches		4.6	4.4	1.1	0																		11.9
7-21 inches		3.2	2.7	1.1	0																		18.8
21-53 inches		357.2	87.0	50.4	50.4	52.7	45.8	41.2	32.1	22.9	14.7	14.0	13.7	12.4	11.5	9.8	8.2	7.3	5.5	4.6	2.3	843.7	493.7
NPK (sod):		3.9	1.1	0																			5.0
0-7 inches		3.7	1.1	0																			12.9
7-21 inches																							4.8
21-53 inches																							

¹ Expressed in terms of dry weight of soil.

² Trace.

1 PERCENT CITRIC ACID (pH 2.2)

Description of soil	Phosphoric acid in successive leaching indicated										Total P_2O_5 in all leachings	Absolute amounts
	1	2	3	4	5	6	7	8	9	10		
Soil before trees were planted:	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	$P, p, m.$	Grams
0-7 inches	125.4	53.2	38.9	20.9	19.0	17.5	16.2	11.7	13.2	13.0		194.8
7-21 inches	22.5	13.0	(2)									41.9
21-53 inches	20.0	8.0	(2)									75.3
Check (sod):	138.0	72.5	45.4	38.2	33.6	30.5	26.3	18.0	10.0	8.0		246.0
0-7 inches	17.4	7.1	0	0								24.5
7-21 inches	21.0	9.8	3.6	0								97.9
21-53 inches												
Check (cultivation):	142.0	85.6	52.1	33.8	30.5							63.6
0-7 inches	28.2	15.3	7.9	2.5	0							37.8
7-21 inches	21.0	10.0	6.8	0								101.7
21-53 inches												
NPK (sod):	1,006.3	176.7	119.7	87.4	68.4	60.8	60.8	55.0	38.0	22.8		1,785.9
0-7 inches	21.4	10.0	6.2	3.7	0							41.3
7-21 inches	21.0	11.0	6.0	0								38.0
21-53 inches												
NPK (cultivation):	902.4	138.7	90.6	70.2	53.2	51.4	45.5	40.1	38.7	25.4		1,456.5
0-7 inches	38.7	21.3	21.1	20.4	10.2	6.2	0					120.6
7-21 inches	21.1	10.2	7.1	(2)								36.3
21-53 inches	19.0											97.6

0.2 N NITRIC ACID (pH 0.7)

Soil before trees were planted:													Phosphoric acid in successive leaching indicated										Total P_2O_5 in all leachings	Absolute amounts in all leachings
													Phosphoric acid in successive leaching indicated											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		Grams		
0-7 inches	56.0	62.6	45.6	30.4	32.2	30.4	22.4	20.0	19.1	18.0												336.7		
7-21 inches	13.0	8.0	(3)																			21.0		
21-53 inches	15.2	8.0	(3)																			23.2		
Check (sod):	61.6	60.8	57.0	49.4	45.6	38.0	32.3	30.4	30.4	22.8												431.3		
0-7 inches	10.0	6.8	(3)																			16.8		
7-21 inches	10.0	7.5	(3)																			17.5		
21-53 inches																								
NPK (sod):	1,178.0	135.0	91.2	60.8	41.8	39.9	22.9	20.4	20.4	17.1												952.3		
0-7 inches	25.2	7.2	(3)																			38.2		
7-21 inches	21.7	7.5	(3)																			32.5		
21-53 inches																						87.1		

¹ Expressed in dry weight of soil.

² Trace.

³ Percolation was not carried further.

DISCUSSION AND INTERPRETATION OF RESULTS

EXTENT OF AND DIFFERENCES IN THE DOWNWARD MOVEMENT OF PHOSPHORUS IN THE TWO CULTURE SYSTEMS AS DETERMINED BY TOTAL PHOSPHORUS CONTENT

The weights of each of the three soil horizons are: Surface 1,290 pounds, subsurface 2,600 pounds, and subsoil 5,930 pounds. The absolute amounts of phosphoric acid can, therefore, be calculated. They are shown in table 2.

The absolute amounts of phosphoric acid in the surface soil of correspondingly treated cylinders under the two systems is very much greater under sod than under cultivation, but the condition is reversed in the subsurface layer. The quantities of residual applied phosphoric acid expected in the surface soil at the end of the experiment can be approximately calculated from the data of table 2 together with the known amounts applied and absorbed by the trees from the added phosphate (46). The data so calculated indicate that a downward movement of phosphorus into the subsurface occurred in all the cylinders under cultivation and that no movement into the subsurface occurred under sod. The absolute amounts of phosphoric acid which moved into the subsurface (7 to 21 inches) layer in the cylinders under cultivation were: NPK, 117 g; PK, 113 g; and P, 99 g—equivalent to 11.5 and 9.5 percent of the amounts added in the NPK- and P-treated cylinders.

The difference in the behavior of the cylinders under cultivation with respect to phosphorus movement as well as with respect to the movement of nitrogen (47) is interesting. For a better understanding it will be necessary to refer to the distribution and condition of the nitrogen in these same cylinders (47). In order to explain the accretion of nitrogen as nonnitric nitrogen in the subsoil (21 to 53 inches) under cultivation (no accretion of nitrogen occurred under sod), three explanations were advanced, viz, (1) assimilation of applied nitric nitrogen by micro-organisms, (2) peptization of organic nitrogen by sodium nitrate in the surface soil and subsequent movement into the subsoil, and (3) the greater root system of the apple trees under cultivation as compared with that under sod.

In searching for an explanation of this movement of phosphorus (and also of nitrogen) in the cylinders under cultivation (but not from under sod) the first explanation given above would a priori be eliminated. The third explanation would account for only a fraction of the phosphoric acid that moved into the subsurface. An additional explanation has recently been suggested, viz, that the living grass roots themselves absorbed and held enough phosphorus as it became available to reduce the amount that moved downward to a negligible quantity. This would be a consequence of greater downward movement of water under cultivation than under sod because there would be less transpiration and, therefore, less upward movement. These causes, no doubt, would be a factor but a minor factor because the phosphoric acid content of the grass in the sod cylinders would account for less than one twenty-fifth of the phosphoric acid that moved into the subsurface layer of the cylinders under the cultivation system.

The second explanation above, viz, that of "peptization" by the organic matter, remains to be considered. There is sufficient experimental evidence (29, 30, 36) to show that colloiddally dispersed humic

acids (formed by bacterial activity) function in making the soil phosphoric acid more soluble. Ramann (36) gives many instances of soils being impoverished in phosphorus under such conditions. Dicalcium phosphate (CaHPO_4) is relatively quickly decomposed by humic acids with the formation of calcium humates and free phosphoric acid, the fate of which depends on the amount of sesquioxides present (30). The action of the humic acids may be expressed as a "deactivation" of the sesquioxides (9, 10).

The relative immobility of phosphorus in the cylinders under sod is in accord with the results obtained in many other long-continued field experiments on the heavier types of soils (14, pp. 12-124; 43, 49), where little movement of applied phosphates occurred below plow depth, except where certain salts (14) or stable manures (43) were added. In orchards under sod, penetration of phosphates in heavy clay soils may not exceed the first inch (42). On the other hand, on loosely compacted soils evidence of phosphate movement below the first foot has been obtained (42, 43, 51). Phosphoric acid has not been found in the leachates from lysimeter experiments (22, 31). In prairie soils a gradual translocation of phosphorus from the subsurface to the surface horizons has been noted (1).

CONDITION OF THE PHOSPHORUS

SOURCE OF THE PHOSPHORUS AND OF THE IRON AND ALUMINUM IN THE HAGERSTOWN SERIES

Frear and White (19) in an examination of the available phosphoric acid on the grasslands of the Jordan fertilizer plots found only 6.4 percent of the total amount of phosphoric acid present soluble by the Dyer method (one extraction only), and observed that this indicated the absence of apatite. The very small amounts of phosphoric acid dissolved from the original soil by all solvents (table 3) point to the absence of apatite. This is further confirmed by the mineralogical examination of Honess (44, footnote 2). Limonite comprises two-thirds of the accessory species in the fine sand fraction (0.2 to 0.1 mm) of the surface soil, and it is probable that the source of phosphorus in this soil is as an impurity in the limonite ($\text{Fe}_2\text{O}_3 \cdot n\text{H}_2\text{O}$), known to contain admixtures of other elements including phosphorus (5). Limonite has a high "fixing" capacity for phosphorus (15).

During the weathering processes aluminum compounds are in part transferred into various hydrated silicates. The aluminum of this soil is derived from the feldspars, which constitute about 50 percent of the fine-sand fraction and a large proportion of the silt fraction. Other sources of aluminum are the amphiboles and pyroxenes present as accessory species. There is no evidence of the presence of bauxite in this soil (44). The iron is present as iron oxides mostly as limonite.

PERCOLATION DATA

The leaching experiments were limited to an examination of (1) the original soil before planting (the pH of which was 6.8), (2) the check cylinders under sod and cultivation (the pH of which was 6.65 and 6.60, respectively), (3) the NPK cylinders also under sod and cultivation (the pH of which was 6.50).

The results of the percolation experiments for the three horizons of the foregoing soils are given in table 3.

CONDITION OF THE RESIDUAL PHOSPHORUS IN THE SURFACE HORIZONS

In the case of those solvents having a pH value of 2.2 and below the concentration of the solution with respect to phosphoric acid falls with each successive extraction, and with all solvents tends toward a constant level. At the stage indicated by reduction to this constant level there must exist a phosphate of such low solubility that the amount going into solution at each extraction is independent of the mass present in the soil. But with none of the solvents do the amounts going into solution follow a logarithmic law of decrement in either the phosphate-treated or the untreated soils—a consequence of the existence of more than one phosphate in both soils. In the phosphate-treated soils nitric and citric acids have brought out 60 to 70 percent of the total phosphoric acid in the surface 0 to 7 inches in the first extract, indicating the presence of at least one comparatively easily soluble phosphate which dissolves in proportion to the mass of it present in the soil.

Inasmuch as there is no evidence in these laboratory experiments that the added monocalcium phosphate has effected any significant changes in the solubility of the native phosphorus, we can proceed to ascertain the condition of the added phosphate by comparing the amounts removed by the different solvents after an equal number (ten) of leachings, allowance being made for the amounts extracted from the check cylinder by the respective solvents. The amount dissolved from the unfertilized soil (i. e., the check cylinder) must be taken into consideration because the quantities of phosphoric acid removed by different acids from the check cylinder may bear (as in this experiment) no constant relation to that extracted from the fertilized soil. The results for the NPK cylinder in sod are shown in table 4.

TABLE 4.—Phosphoric acid (P_2O_5) dissolved from the surface soil of the NPK cylinders expressed as a percentage of the residual of the amount applied

IN SOD		
Solvent	Indicated method of calculation	Percentage of residual applied P_2O_5 dissolved
Distilled water.....	$\frac{353.1-5.0}{1.039} \times 100 =$	33.5
0.1 N acetic acid.....	$\frac{441.4-13.6}{1.039} \times 100 =$	41.2
0.002 N H_2SO_4	$\frac{544.6-13.5}{1.039} \times 100 =$	51.1
0.2 N HNO_3	$\frac{952.2-252.4}{1.039} \times 100 =$	67.3
0.17 N citric acid.....	$\frac{1,045.0-246.0}{1.039} \times 100 =$	76.9
IN CULTIVATION		
Distilled water.....	$\frac{285.2-6.0}{917} \times 100 =$	30.4
0.002 N H_2SO_4	$\frac{534.3-21.9}{917} \times 100 =$	55.9
0.17 N citric acid.....	$\frac{852.2-246.0}{917} \times 100 =$	66.1

The action of a dilute acid upon soil is not a simple action of attack of an easily soluble phosphate followed by a more difficultly soluble phosphate (23, 24), nor, indeed, as already pointed out, is the action of a dilute acid constant (38). Rather does extraction with the different weak acids result in a condition of equilibrium which modifies the initial partition of phosphoric acid in the solid and liquid phases. If the amount of phosphoric acid in the liquid phase is sufficiently high the solution will give up phosphoric acid to the soil, and vice versa. There must exist, therefore, a critical concentration (9); this critical concentration is different for different acids, low for acetic and nitric acids and high for citric acid (10). This critical concentration depends on the phosphorus reserves of the soil and also on the "fixing" power. It is also clear from Russell and Prescott's (38) experiments that no definite fraction of the phosphates present is obtained by extraction with dilute acids but only that part expressed as the difference between the amount dissolved and absorbed. For all these reasons it is not possible by such means to separate each of the different phosphorus compounds—to separate, for example, the phosphates of calcium from one another—by treatment of such an adsorption substrate as a soil with different dilute acids, as some investigators have attempted to do.

One may, however, from the data of table 4 conclude that one-third of the residual applied phosphoric acid from the NPK cylinder in sod remained (under conditions of continuous leaching) in a condition soluble in water, one-third has been transformed into somewhat less soluble compounds, and one-third (100–67 percent) into very difficultly soluble compounds.

The results for the surface 0 to 7 inches of the phosphate-treated cylinder under cultivation are of the same order of magnitude for the distilled water and sulphuric-acid extractions, viz, 30.4 and 55.9 percent, respectively. But the percentage of the amount residual in the surface layer under cultivation dissolved by citric acid is only 66.1 compared with 76.9 from the correspondingly treated cylinder under sod. This apparently anomalous result will be reserved for later discussion.

The first and second distilled water percolates of the surface 0 to 7 inches of the treated cylinders, both in the sod and in the cultivation systems, contain as much as 13.5 and 7.9 percent of the residual amounts applied during the course of this experiment. This fact suggests that the amounts added may have been more than sufficient to saturate the aluminum and iron hydroxides in these surface layers.

The difficultly soluble compounds into which the soluble phosphate has in part been transformed must be more difficultly soluble than chemically pure ferric phosphate. This may be deduced from the results given in table 5, in which are shown the results of percolation experiments in which chemically pure ferric phosphate, aluminum phosphate, and tricalcium phosphate were each mixed with the original soil in amounts equal to the application of phosphoric acid to the phosphate-treated cylinders. Table 5 gives the percentages of phosphoric acid removed by the different solvents.

TABLE 5.—Phosphoric acid (P_2O_5) removed by the percolation method from c. p. ferric phosphate, c. p. aluminum phosphate, and c. p. calcium phosphate mixed with the "original" soil

Solvent	pH	FePO ₄ ·AlH ₂ O		AlPO ₄		Ca ₃ (PO ₄) ₂	
		Extractions	Removed	Extractions	Removed	Extractions	Removed
		Number	Percent	Number	Percent	Number	Percent
Distilled water.....	5.5	10	0	10	14	10	21
0.002 N sulphuric acid.....	3.0	10	0	10	32.6	4	100
0.1 N acetic acid.....	2.8	10	7.9	10	66	6	100
0.17 N citric acid.....	2.2	8	100	10	100	2	100
0.2 N nitric acid.....	0.7	15	100	10	100	2	100

The results are similar to those obtained by Heck (26) and also are in accord with Gaarder's scheme (21). Relative to the comment given on page 333, it may be added that the nearest information that can be obtained with respect to the proportions of the various phosphates existing in the phosphate-treated cylinders is through a comparison of the data of table 5 with those of table 4.

CONDITION OF THE PHOSPHORUS IN THE LOWER HORIZONS

Neither water nor 0.002 N sulphuric acid leached out any phosphoric acid from the original soil. The native phosphorus of these soils is, therefore, not soluble in these solvents. Citric acid removed somewhat more than nitric acid from the lower layers of the original soil.

Percolation with water also removed only minute quantities from the lower horizons of the phosphate-treated (and the untreated) cylinders. One-percent citric acid has dissolved greater quantities of phosphoric acid than 0.2 N nitric acid.

The condition of the phosphoric acid that moved from the surface soil into the subsurface of the phosphate-treated cylinder under cultivation is easily traced. Water removed none of it; Truog's solvent dissolved approximately 20 percent and citric acid about 80 percent of it.

EFFECT OF THE GROWTH OF TREES ON THE SOLUBILITY OF THE NATIVE PHOSPHORUS

More phosphoric acid was dissolved from the surface soil of the check cylinders than from the original soil by all solvents. The increase is very marked with citric and also with nitric acid; the relative increases are 51.2 and 55.4 g, respectively, for the cylinder under sod. Inasmuch as these increments are much greater than the amounts of phosphoric acid contributed in the cover crops (p. 322), the native phosphorus of the soil must have been made more soluble during the growth of the trees.

THE ORGANIC PHOSPHORUS

A number of methods have been proposed for determining the organic phosphorus of soils, but none has yet met the criteria of validity indicated by Frear and White (20) in 1911. In these cylinder experiments the organic phosphorus of even the phosphate-treated plots under cultivation as determined by Schollenberger's method

(41) represented only about 1 percent of that applied as monocalcium phosphate. The organic phosphorus is, therefore, relatively insignificant as compared to the amount of phosphoric acid applied.

THE "FIXING" CAPACITY OF SOILS

The writer has determined the fixing capacity of the original Hagerstown soil by many of the different methods proposed to determine this property (15, 16, 18, 25). It is unnecessary, however, to record the results, inasmuch as no additional information was secured beyond that already recorded—a fact which lends further support to the suggestion (p. 333) that the amounts of phosphate applied during the course of this experiment were more than sufficient to saturate the iron and aluminum hydroxides. The fixing capacity of any one soil type is, as already indicated, a relative and not an absolute property; the amount retained is conditioned not only by the concentration of the phosphate ions and the concentration with respect to other elements, especially those of hydrogen, silica, iron, aluminum, calcium, and magnesium (9, 32), but also by temperature and by time (15, 39, 56). The results for the same soil will, therefore, differ with the laboratory procedure with respect to the latter factors. If the fixing capacity of different soil types is to be compared, the method described by Demolon and Barbier (10) should receive the attention of investigators.

The ability to fix phosphorus cannot be unrelated to the form of the phosphate. Wrangell (52) is probably correct in maintaining that the concentration of the soil solution with respect to phosphate ions depends to a greater extent upon the absorbing capacity of the soil than upon the structure and composition of the applied phosphate, for the soil solution has, in all cases examined by Wrangell, a greater concentration with respect to PO_4 ions when treated with tricalcium phosphate (and also rock phosphate) than with monocalcium phosphate. Deductions, however, relative to the availability of phosphates cannot be made from such observations, for, although relatively insoluble phosphates are fixed more slowly than the more soluble phosphates, they are less efficient in supplying available phosphorus to plants (39)—a consequence apparently of solid phase feeding.

SUMMARY

The distribution of phosphoric acid (P_2O_5) in three horizons at the conclusion of an experiment lasting 6½ years on a Hagerstown clay loam soil, contained in cylinders planted to apple trees, and treated with different combinations of sodium nitrate, monocalcium phosphate, and potassium sulphate, are given in percentage and in absolute amounts.

In all the phosphate-treated cylinders the total (i. e., absolute) amounts of phosphoric acid of the surface soil was greater in the cylinders under sod than in those under cultivation. But in the subsurface layer (7 to 21 inches) the absolute amounts of phosphoric acid present were considerably smaller in the cylinders under sod than in those under cultivation. The downward movement of phosphoric acid into the subsurface layer in the cylinders under cultivation is equivalent approximately to 10 percent of the phosphoric acid added.

There is no evidence of movement of phosphoric acid into the sub-surface layer in the cylinder under sod and none into the subsoil (21 to 53 inches) of the cylinders either under cultivation or under sod.

An explanation advanced is that the differences in the movement of phosphorus in the two culture systems is for the most part the result of the mobilizing effect of humic acids.

A simple and compact percolation apparatus is described by the use of which the quantities of phosphoric acid removed in successive leachates from the original soil before the trees were planted and in the soils from untreated and phosphate-treated cylinders by various weak acid solvents were obtained. The results of these extractions are as follows:

In the phosphate-treated cylinders at least one moderately soluble phosphate was present which dissolves in proportion to the mass of it in the soil.

Not a trace of phosphoric acid was found in the leachates from any horizon of the original soil (soil before trees were planted) either by distilled water (pH 5.5) or by 0.002 normal sulphuric acid (pH 3.0).

Thirty-three percent of the phosphoric acid applied to the phosphate-treated cylinders was still soluble in distilled water at the end of the 7-year experiment. One-third remained in a condition of moderate solubility and one-third was converted into basic iron and aluminum compounds more difficultly soluble than ferric phosphate.

There was a conversion into difficultly soluble phosphates of that portion of the applied phosphoric acid that had moved into the sub-surface layer in the cylinder under cultivation.

A marked increase was observed in the availability of the phosphoric acid of the soil in the check cylinders over that of the original soil.

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VEGETABLE PROTEIN IN TURKEY RATIONS



A REPRESENTATIVE GROUP OF 5-WEEK-OLD TURKEYS RECEIVING SOYBEAN OIL MEAL
IN THE RATION

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Vegetable Protein in Turkey Rations

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IN 1929 the Pennsylvania Agricultural Experiment Station conducted a study which was reported in Bulletin 250, *Feed Consumption and Costs in Raising Turkeys*. Bronze and White Holland poults were fed, until 20 weeks of age, an all-mash ration containing 16½ per cent protein. Fresh drinking water and liquid skim milk were always available. Scratch grain was supplied after the birds were 20 weeks of age. This provided the poults with an opportunity to adjust the protein intake. Sufficient liquid skim milk was consumed during the first two weeks to maintain a total protein intake of 26 per cent. From the third to the sixteenth weeks the birds maintained a protein level intake of approximately 20 per cent. By the twenty-fourth week, the protein intake had decreased to approximately 15 per cent due to the consumption of scratch grain. The high protein requirements of turkey poults during the first weeks of their lives pointed to the need for further work with various levels and sources of protein.

In 1930² five groups of turkeys were fed for 24 weeks as follows:

- Group 1. A 24 per cent protein mash during the entire period, and with access to a sunporch.
- Group 2. A 24 per cent protein mash for the first eight weeks; a 20 per cent protein mash from 9 to 16 weeks and a 16½ per cent protein mash from 17 to 24 weeks. Confined for 24 weeks, with access to a sunporch.
- Group 3. The same ration as Group 2. Confined the first eight weeks with access to a sunporch and then placed on range for the duration of the experiment.
- Group 4. A 20 per cent protein mash for 24 weeks. Confined with access to a sunporch.
- Group 5. A 16½ per cent protein mash for 24 weeks. Confined with access to a sunporch.

There was greater feed consumption in the groups which received the higher levels of protein. Better growth during the first eight weeks was obtained from a 24 per cent level of protein. There was little difference in the efficiency of the various levels of protein studied as measured by rate of growth after the poults were eight weeks of age. A higher percentage of mortality occurred in the group receiving the 16½ per cent level of protein. This study indicated that turkey poults require a high protein intake during the early part of their lives.

As a result of these investigations the Pennsylvania State College has recommended a mash containing 24 per cent protein for the first eight weeks and a mash containing 20 per cent protein thereafter. This mash should be available

¹ Publication authorized August 5, 1935.

² Directed by E. M. Funk, now of the Missouri Agricultural Experiment Station, until July 1, 1930.

to the turkeys at all times. Scratch grain should be supplied in hoppers from 12 weeks of age until the birds are marketed. This schedule of feeding provides definite protein reduction as the birds increase in age. Poultrymen have adopted a high protein turkey ration quite generally and the results have been highly satisfactory. It is now generally accepted that for optimum growth, turkey rations should contain a higher percentage of protein than chicken rations.

Object

Heretofore turkey rations of high protein content have been secured by the use of liberal quantities of animal protein concentrates, such as meat scraps, dried milk, and fish meal. These are comparatively expensive. Hence, there has been demand for a study of vegetable protein substitutes that will lower the cost of the rations without reducing their efficiency. Soybean oil meal and corn gluten meal are high in protein and are produced in large quantities. In normal times these products sell at a lower cost on the basis of protein content than animal protein concentrates. The value of animal protein in poultry rations has been shown by many investigators. The purpose in this investigation was not to eliminate animal protein but to supplement a portion of it with less costly vegetable protein concentrates.

Since animal protein concentrates carry considerable quantities of mineral matter, particularly calcium and phosphorus, it appeared desirable to compensate the mineral loss, when vegetable protein concentrates were substituted, by additions of calcium and phosphorus in the form of bone meal, calcium carbonate, or both. A basal ration of 24 per cent protein, containing only animal protein concentrates, was used as a basis of comparison. When changes in the basal ration were made, adjustments in the protein, calcium and phosphorus levels were made also so that all the rations were approximately equal in these components.

Experiments in 1933

One hundred and eighty-four day-old turkey poults of the Bronze and White Holland varieties, hatched July 18, 1933, were divided into eight groups of 23 poults each and maintained under experiment for 16 weeks. Each group contained 13 White Holland poults and 10 Bronze poults. The groups were confined in a long brooder house in pens identical in size, lighting, ventilation, heating, and equipment. Each pen was 12 by 20 feet and was equipped with a screened sunporch 9 by 10 feet, the base of which was covered with 1-inch mesh wire flooring.

Table 1 shows the composition of the various rations fed to the eight groups. Table 2 shows, in condensed form, the basal ration and the vegetable protein replacements. The rations contained approximately 24 per cent crude protein. Five years of experience at The Pennsylvania State College, involving studies with several thousand turkeys, have led to the conclusion that this protein content is adequate.

The feed was supplied as mash and was available at all times. Individual weights of all birds were recorded bi-weekly. Records of feed consumption, mortality, and general appearance of the poults were kept throughout the experimental period. Oyster shells and insoluble grit were before the birds at all times. No other supplements were fed.

TABLE 1. COMPOSITION OF TURKEY STARTER RATIIONS, 1933 AND 1934

Group No.	1	2	3	4	5	6	7	8
	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
Dried skim milk	12.00	6.00	6.00	6.00	12.00	12.00	6.00	6.00
Meat scraps	11.00	11.00	5.50	11.00	5.50	11.00	11.00	7.00
Fish meal	11.00	11.00	11.00	5.50	5.50	.00	5.50	7.00
Soybean oil meal	.00	5.00	12.00	12.00	14.00	11.00	.00	.00
Corn gluten meal	.00	.00	.00	.00	.00	.00	12.50	16.00
Wheat bran	13.40							
Wheat middlings	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Ground heavy oats	10.00							
Alfalfa meal	5.00							
Salt	1.00							
Cod liver oil	1.00							
Ground yellow corn	21.00	21.75	21.86	21.86	18.11	18.11	21.13	19.46
Steamed bone meal	.00	.17	1.50	1.50	2.68	2.68	1.87	2.54
Ground limestone	.00	.04	.11	.11	.18	.18	.00	.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

TABLE 2. DEDUCTIONS OF ANIMAL PROTEIN CONCENTRATES AND REPLACEMENTS WITH VEGETABLE PROTEIN CONCENTRATES IN THE BASAL RATION, 1933

Group No.	Dried skim milk	Meat scraps	Fish meal	Soybean oil meal	Corn gluten meal
	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
1*	---	---	---	5.0	---
2	6	---	---	12.0	---
3	6	5.5	---	12.0	---
4	6	---	5.5	14.0	---
5	---	5.5	5.5	14.0	---
6	---	---	11.0	---	12.5
7	6	---	5.5	---	16.0
8	6	4.0	4.0	---	---

*This group was fed the basal ration, and no deductions or replacements of animal or vegetable protein concentrates were made.

TABLE 3. AVERAGE BODY WEIGHT PER TURKEY, 1933

Group No.	1	2	3	4	5	6	7	8
	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
Age in weeks								
Day old	.13	.13	.13	.13	.12	.12	.13	.13
2 weeks	.32	.30	.35	.33	.33	.29	.29	.31
4	.71	.65	.74	.73	.73	.67	.66	.70
6	1.46	1.40	1.55	1.46	1.45	1.36	1.32	1.36
8	2.48	2.48	2.80	2.49	2.65	2.49	2.29	2.41
10	3.81	3.60	4.27	3.76	3.98	3.71	3.45	3.67
12	4.99	5.02	5.71	5.35	5.42	5.11	4.85	5.38
14	6.58	6.47	7.23	6.76	6.81	6.42	6.28	6.44
16	8.97	7.85	8.73	8.32	8.20	7.88	7.60	7.60
Day old	.13	.12	.13	.12	.13	.13	.14	.13
2 weeks	.31	.32	.35	.32	.36	.31	.32	.35
4	.68	.68	.76	.71	.76	.76	.76	.75
6	1.50	1.49	1.61	1.55	1.58	1.57	1.59	1.53
8	2.80	2.67	2.92	2.84	2.88	2.81	2.67	2.65
10	3.95	4.17	4.58	4.55	4.46	4.36	4.21	4.22
12	5.58	5.94	6.16	6.15	6.21	6.22	6.08	5.90
14	7.57	8.06	8.32	8.33	7.99	8.17	8.07	7.74
16	9.93	10.34	10.49	10.62	10.00	10.23	10.08	9.52
Ave. body weight at 16 weeks, both sexes	9.00	8.94	9.60	9.47	9.10	9.05	8.84	8.59

Table 3 shows the growth data for each of the eight groups of the 1933 study from day old to 16 weeks of age. The results as recorded are averages weighted for variety.

Growth was essentially the same for all groups. The birds receiving corn gluten meal as a substitute for part of the animal protein showed a much superior finish, especially as to the covering of flesh over the breast. This superior fleshing condition was apparent throughout the growing period and was particularly noticeable when the poults were 10 to 12 weeks of age.

Table 4 shows the feed consumption by groups from one day to 16 weeks. These figures are expressed in pounds of feed consumed per 100 birds.

TABLE 4. FEED CONSUMPTION, 1933
(Pounds of mash consumed per 100 turkeys from 0 to 16 weeks)

Group No.	1	2	3	4	5	6	7	8
Age in weeks	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
0-2	40	36	45	39	42	42	37	41
2-4	82	82	97	94	100	93	99	85
4-6	191	186	203	204	215	217	197	191
6-8	320	315	343	308	316	343	307	259
8-10	410	406	472	442	451	421	434	421
10-12	582	572	605	577	601	643	601	549
12-14	819	727	768	778	757	775	747	716
14-16	944	941	968	943	977	1033	953	903
Total pounds of mash consumed 0-16 weeks	3318	3275	3501	3415	3459	3567	3375	3206

Table 5 gives the mortality data for each group by bi-weekly periods. The average mortality for all groups during the 16-week period was 20.4 per cent. The highest mortality was in groups 7 and 8 where there was 30.1 per cent loss. The groups were too small, however, to warrant giving much weight to these mortality figures. The heaviest mortality occurred during the first 2-week period and probably was not due to the ration.

TABLE 5. MORTALITY DATA, 1933

Group No.	1	2	3	4	5	6	7	8
Age in weeks	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
0-2	0.0	4.3	8.6	8.6	8.6	12.9	12.9	12.9
2-4	4.3	4.3	0.0	0.0	0.0	4.3	8.6	4.3
4-6	4.3	0.0	0.0	0.0	4.3	0.0	0.0	4.3
6-8	0.0	0.0	8.6	0.0	4.3	4.3	0.0	0.0
8-10	8.6	4.3	0.0	4.3	0.0	0.0	4.3	8.6
10-12	0.0	0.0	0.0	0.0	0.0	0.0	4.3	0.0
12-14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14-16	0.0	0.0	0.0	0.0	4.3	0.0	0.0	0.0
Total 0-16 weeks	17.2	12.9	17.2	12.9	21.5	21.5	30.1	30.1

Experiments in 1934

Since the results of 1933 experiments indicated that a portion of the animal protein in the turkey starter could be replaced by soybean oil meal or corn gluten meal, it seemed advisable to repeat this experiment for a longer period using larger groups. On June 12, 1934, 328 turkey poults, one week of age,

were divided into eight groups of 41 poults each. Each group contained 16 White Holland and 25 Bronze poults. The experiment was begun when the poults were one week of age in order to eliminate the occasional abnormal bird not suited for experimental work. The housing conditions were identical with those of the experiment conducted in 1933. The rations for the first 12 weeks were the same as those used in 1933 (Table 1). When the poults were 12 weeks of age, fish meal was replaced with meat scrap and cod liver oil

TABLE 6. COMPOSITION OF RATIONS USED FROM 13 TO 25 WEEKS OF AGE, 1934

Group No.	1	2	3	4	5	6	7	8
Per cent protein	16	16	16	20	20	20	16	16
	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
Dried skim milk	5.00	3.33	1.67	7.00	4.67	2.33	3.33	1.67
Meat scraps	10.00	6.67	3.33	14.00	9.33	4.67	6.67	3.33
Soybean oil meal	.00	5.00	10.00	.00	7.00	14.00	.00	.00
Corn gluten meal	.00	.00	.00	.00	.00	.00	5.00	10.00
Wheat bran	13.00							
Wheat middlings	12.00							
Ground heavy oats	10.00	41.00	41.00	41.00	41.00	41.00	41.00	41.00
Alfalfa meal	5.00							
Salt	1.00							
Ground yellow corn	44.00	43.04	42.07	37.00	37.04	36.07	42.97	41.91
Steamed bone meal	.00	.87	1.74	.00	.87	1.74	1.03	2.06
Ground limestone	.00	.09	.19	.00	.09	.19	.00	.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

TABLE 7. DEDUCTION OF ANIMAL PROTEIN CONCENTRATES AND REPLACEMENTS WITH VEGETABLE PROTEIN CONCENTRATES IN THE BASAL RATION, 1934 (13 WEEKS TO 25 WEEKS)

(12 WEEKS TO 25 WEEKS)						
Group No.	Per cent protein in mash	Scratch grain	Animal protein concentrate deductions per 100 pounds of feed		Vegetable protein concentrate replacements per 100 pounds of feed	
			Dried skim-milk	Meat scraps	Soybean oil meal	Corn gluten meal
			Lbs.	Lbs.	Lbs.	Lbs.
1	16	None	.00	.00	.00	.00
2	16	None	1.67	3.33	5.00	.00
3	16	None	3.33	6.67	10.00	.00
4	20	Ad lib*	.00	.00	.00	.00
5	20	Ad lib*	2.33	4.67	7.00	.00
6	20	Ad lib*	4.67	9.33	14.00	.00
7	16	None	1.67	3.33	.00	5.00
8	16	None	3.33	6.67	.00	10.00

* Scratch grain in hoppers was constantly available.

was omitted from the ration, in order to prevent the possibility of a fishy flavor in the flesh, as had been experienced in previous years.

During the last 12 weeks of the experimental period, the protein levels were reduced, since previous work at this station had shown that lower protein rations may be used advantageously after the early growth period. Throughout the remainder of the growing period, it seemed inadvisable to feed a higher level of protein than that required for satisfactory growth.

During the period 13 to 25 weeks, the composition of the rations was changed (Table 6). The deduction of animal protein concentrates from the

basal ration and the vegetable protein replacements are shown in Table 7. During the period 13 to 25 weeks (Tables 6 and 7) an all-mash ration containing 16 per cent protein was supplied to groups 1, 2, 3, 7, and 8. Group 1 was used as the basal for these groups. Soybean oil meal was added to the rations fed groups 2 and 3 to replace one-third and two-thirds respectively of the animal protein concentrates. Group 1 also served as a basal for groups 7 and 8. In group 7, one-third of the animal protein was replaced by corn gluten meal. Two-thirds of the animal protein was replaced by corn gluten meal in group 8. A 20 per cent protein mash was fed to groups 4, 5, and 6, during the last 12 weeks of the experimental period, because the addition of scratch grain made it possible for the turkeys to adjust their protein intake.

The scratch grain supplied to these groups was composed of 50 per cent cracked yellow corn, 25 per cent wheat, 15 per cent oats, and 10 per cent barley. These three pens were placed on a mash and scratch grain combination in order to more closely approximate farm feeding conditions. Group 4 served as a basal for this series of three pens and groups 5 and 6 had one-third and two-thirds respectively of their animal protein concentrate replaced by soybean oil meal.

In groups 1, 2, 3, 7, and 8, a level of 16 per cent protein was maintained. Sufficient calcium and phosphorus were added to groups 2, 3, 7, and 8 in the form of bone meal and calcium carbonate to maintain the same levels of calcium and phosphorus in all five groups.

The growth data for turkeys in the 1934 experiment (1 to 25 weeks) are given in Table 8. All growth data were analyzed statistically. The results show no significant differences among the various groups.

TABLE 8. AVERAGE BODY WEIGHT PER TURKEY, 1934

Group No.	1	2	3	4	5	6	7	8
Age in weeks	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
Females								
1 week	.19	.18	.19	.18	.19	.19	.19	.18
3	.49	.47	.48	.44	.45	.42	.50	.43
5	1.19	1.18	1.20	1.05	1.10	.98	1.17	1.01
7	2.09	2.07	2.06	1.83	1.91	1.70	2.04	1.79
9	3.32	3.34	3.26	3.02	3.15	2.83	3.30	2.98
11	4.09	4.07	4.56	4.31	4.28	4.05	4.09	4.17
13	5.95	6.20	5.54	5.46	5.69	5.42	6.09	5.76
17	8.30	8.25	7.73	7.94	7.96	7.75	8.42	7.69
21	10.44	10.27	9.91	10.22	9.96	9.88	10.49	9.80
25	11.70	11.69	11.63	11.61	11.64	11.50	11.97	11.48
Males								
1	.21	.20	.21	.19	.20	.19	.20	.20
3	.55	.52	.55	.48	.49	.46	.54	.51
5	1.36	1.37	1.37	1.21	1.22	1.10	1.30	1.21
7	2.41	2.44	2.39	2.12	2.23	1.94	2.30	2.20
9	3.89	4.03	3.92	3.58	3.81	3.65	3.86	3.67
11	5.92	5.81	5.61	5.22	5.41	5.03	5.63	5.28
13	7.47	8.02	7.19	6.97	7.39	7.04	7.63	7.50
17	11.06	11.32	11.00	10.88	11.02	10.85	10.89	10.27
21	14.78	15.09	14.74	14.86	14.93	14.82	14.40	13.68
25	17.46	17.82	17.44	17.51	17.93	17.78	17.30	16.36
Ave. body wt. at 25 weeks, both sexes	11.58	11.76	11.54	11.56	11.78	11.64	11.64	11.32

Table 9 shows the feed consumption from 1 to 25 weeks of age for the eight groups used in the 1934 study. In those groups which received scratch grain, there was a definite tendency to consume less scratch grain as the percentage of vegetable protein concentrates was increased.

Table 10 gives the mortality data for each group. The mortality was relatively low and apparently was not influenced by the ration fed. The average mortality for all groups from 1 to 25 weeks of age was 10.4 per cent.

In the 1933 studies, the birds which received corn gluten meal showed a much superior finish, as characterized by the covering of flesh over the breast. In 1934, two observers handled the birds at 25 weeks of age and scored them

FIG. 1. CLASSIFICATION OF DRESSED TURKEYS FOR FLESHING CONDITION
LEFT TO RIGHT—GOOD, MEDIUM, POOR

for market condition. They were classified into three grades: poor, medium, and good finish (Table 12).

Table 13 shows the pounds of feed required by the various groups to produce a pound of gain.

Discussion of Data

A study of the growth data obtained in the experiments of 1933 and 1934 shows that a portion of the animal protein can be replaced by soybean oil meal or corn gluten meal without unfavorable results on the growth or condition of the birds. In normal years, both of these concentrates sell for a lower price, on the basis of protein content, than meat and fish meals. Dried milk invariably sells at a higher price than any other protein concentrate. Because of the high biological value of milk protein and the high vitamin G content of milk, it was considered desirable that part of the protein should be derived from this source in all of the experimental rations. In the 1933 and

1934 studies, it was possible to replace 50 per cent of the milk, meat scraps, or fish meal with either of the two vegetable proteins studied and still obtain highly satisfactory results. It was also possible to replace half of any two of the three animal protein concentrates used. Tables 3 and 8 indicate satisfactory growth where such changes were made. In groups 3, 4, 5, and 7 (Table 2) one-half of two of the animal protein concentrates used were replaced by soybean oil meal or corn gluten meal.

TABLE 9. FEED CONSUMPTION PER 100 TURKEYS, 1934

Group No.		1	2	3	4	5	6	7	8
Age in weeks	Kind of feed	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
1-3	Mash	77	72	68	69	74	68	76	76
3-5	Mash	167	168	170	159	169	144	164	151
5-7	Mash	280	263	27	258	257	248	271	274
7-9	Mash	424	401	401	409	411	425	402	410
9-11	Mash	543	529	527	513	477	535	549	526
11-13	Mash	698	691	610	638	657	651	688	659
13-17	Mash	1672	1550	1591	1192	1340	1400	1698	1499
13-17	Grain	-----	-----	-----	307	184	295	-----	-----
17-21	Mash	2177	2067	2121	1133	1270	1322	2049	2006
17-21	Grain	-----	-----	-----	809	611	587	-----	-----
21-25	Mash	2207	2238	1997	756	778	846	2198	2267
21-25	Grain	-----	-----	-----	1408	1155	1049	-----	-----
Total mash 1-25 wks.		8345	7979	7762	5127	5424	5639	8095	7847
Total grain 1-25 wks.		-----	-----	-----	2524	1950	1841	-----	-----
Total feed 1-25 wks.		8345	7979	7762	7651	7374	7480	8095	7847

TABLE 10. MORTALITY DATA, 1934

Group No.	1	2	3	4	5	6	7	8
Age in weeks	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
1-3	0.0	2.4	0.0	4.8	2.4	0.0	4.8	0.0
3-5	0.0	2.4	0.0	0.0	0.0	0.0	2.4	4.8
5-7	0.0	2.4	0.0	0.0	2.4	0.0	0.0	0.0
7-9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9-11	0.0	0.0	0.0	0.0	0.0	2.4	0.0	0.0
11-13	0.0	2.4	2.4	0.0	2.4	2.4	0.0	0.0
13-17	2.4	0.0	9.6	2.4	0.0	0.0	2.4	4.8
17-21	4.8	2.4	0.0	0.0	0.0	2.4	2.4	2.4
21-25	2.4	0.0	0.0	0.0	0.0	0.0	2.4	0.0
Total 1-25 weeks	9.6	12.0	12.0	7.2	7.2	7.2	14.4	12.0

Saving in Cost of Rations.—The object of this study was to determine if part of the high-priced animal protein concentrates could be replaced with less costly vegetable protein concentrates. Obviously, the cost figures reported have little value because of fluctuation in feed prices. Table 11 shows the difference in price between the protein concentrates studied and can be used in calculating the saving in feed costs when the various substitutions are made. During 1933, a saving of approximately \$5.50 per ton could be effected. Because of drought conditions and the consequent heavy sale of livestock in the summer of 1934, the price of meat protein was relatively low in compari-

TABLE 11. APPROXIMATE SAVINGS IN COST PER TON OF STARTING MASH WHEN SOYBEAN OIL MEAL OR CORN GLUTEN MEAL REPLACES 100 POUNDS OF THE DRIED MILK AND 100 POUNDS OF THE MEAT OR FISH MEAL IN ONE TON OF FEED

When the price of dried milk exceeds the price of vegetable protein per ton by:	When the price of meat or fish meal exceeds the price of vegetable protein per ton by:									
	0	\$5.00	\$10.00	\$15.00	\$20.00	\$25.00	\$30.00	\$35.00	\$40.00	\$50.00
0	0	.25	.50	.75	1.00	1.25	1.50	1.75	2.00	2.25
\$10.00	.50	.75	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75
20.00	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25
30.00	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75
40.00	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25
50.00	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50	4.75
60.00	3.00	3.25	3.50	3.75	4.00	4.25	4.50	4.75	5.00	5.25
70.00	3.50	3.75	4.00	4.25	4.50	4.75	5.00	5.25	5.50	5.75
80.00	4.00	4.25	4.50	4.75	5.00	5.25	5.50	5.75	6.00	6.25
90.00	4.50	4.75	5.00	5.25	5.50	5.75	6.00	6.25	6.50	6.75
100.00	5.00	5.25	5.50	5.75	6.00	6.25	6.50	6.75	7.00	7.25
110.00	5.50	5.75	6.00	6.25	6.50	6.75	7.00	7.25	7.50	7.75
120.00	6.00	6.25	6.50	6.75	7.00	7.25	7.50	7.75	8.00	8.25

son with vegetable protein; this prevented as large a saving as was possible in 1933.

During the second 12-week period of the 1934 study only two animal protein supplements were used, fish meal being eliminated after the poults were 13 weeks of age. Whenever vegetable protein concentrates were substituted for part of the animal protein concentrates, they replaced either one-third or two-thirds of the total animal protein present in the basal ration. The same proportionate part of each of the two animal protein concentrates was replaced in all instances.

Table 12 shows that the highest percentage of birds in good finish at 25 weeks of age were in those groups where corn gluten meal was fed. Since this was noted in both 1933 and 1934, it is worthy of further consideration. In 1934, scratch grain was fed to three of the groups. The birds showed a preference for corn as long as any remained in the scratch mixture. The comparatively large quantities of corn in the scratch grain did not produce the high finish that was obtained in the two groups which received corn gluten meal in an all-mash ration.

One of the greatest problems of the turkey grower is to produce a well-fleshed carcass at marketable age. Since the improvement of fleshing condition was apparent in groups receiving corn gluten meal during both years of the

TABLE 12. CLASSIFICATION OF 25-WEEK OLD TURKEYS FOR FLESHING CONDITION, 1934

Group No.	Number of birds	Poor finish		Medium finish		Good finish	
		Number	Per cent	Number	Per cent	Number	Per cent
1	37	7	18.9	13	35.1	17	45.9
2	36	7	19.4	16	44.4	13	36.1
3	36	5	13.9	9	25.0	22	61.1
4	39	5	12.8	16	41.0	18	46.2
5	36	0	0.0	15	41.7	21	58.3
6	38	3	7.9	15	39.5	20	52.6
7	34	1	2.9	1	2.9	32	94.2
8	35	2	5.7	7	20.0	26	74.3

experiment and as early as 10 weeks of age, there is a possibility that this particular source of protein will meet the requirements of producers who are attempting to finish turkey broilers or are interested in marketing a portion of their turkey crop prior to the Thanksgiving season. This source of protein also offers possibilities for finishing late-hatched poults in order to market them during the Christmas season, when there is always a demand for small, well-finished birds.

TABLE 13. THE RELATION OF FEED CONSUMPTION AND GROWTH, 1934

Group No.	1	2	3	4	5	6	7	8
Feed to produce one pound of gain								
Age in weeks	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
1-5	2.20	2.22	2.22	2.45	2.44	2.49	2.50	2.42
5-9	3.03	2.74	2.99	3.06	2.81	3.39	2.89	3.04
9-13	4.60	3.55	4.18	3.93	3.75	3.91	3.74	3.45
13-17	5.76	5.70	5.18	4.64	5.39	5.60	6.29	6.26
17-21	7.59	7.08	7.26	6.24	6.60	6.70	7.40	6.84
21-25	12.14	10.46	9.12	10.45	8.51	9.15	10.04	10.08
Ave. 1-25 wks.	5.88	5.42	5.45	5.29	5.19	5.44	5.65	5.54

Rations and Feeding Practices Suggested

	24% Protein turkey starters		20% Protein turkey developers	
	Using animal protein concentrates	Using animal and vegetable protein concentrates	Using animal protein concentrates	Using animal and vegetable protein concentrates
Yellow corn meal	280	260	310	270
Wheat bran	100	100	150	150
Wheat middlings	100	100	150	150
Ground heavy oats	100	100	100	100
Alfalfa meal	50	50	50	50
Dried milk	100	50	50	40
Meat scraps	150	150	170	70
Fish meal	100	50
Salt	10	10	10	10
Cod liver oil	10	10
Soybean oil meal or Corn Gluten meal*	...	100	...	140
Calcium carbonate	...	20	10	20
	1000	1000	1000	1000

* Soybean oil meal or corn gluten meal may be used interchangeably.

According to Meyer and Hetler³ corn gluten may contain much more vitamin A than the corn from which the gluten is made. It may be possible that the high vitamin A content of corn gluten meal was responsible for the large percentage of birds which was classified in the highest grade. In further ex-

³ Jour. Agr. Res. 39: 10, pp. 767-780, 1929.

periments, corn gluten meal and soybean oil meal will be used separately and in combination with each other.

The pounds of feed required to produce a pound of gain over the entire period is not essentially different in any of the groups (Table 13). Those which received vegetable protein supplements or scratch grain additions made more efficient use of their feed during the last eight weeks of the study.

At day old, feed either starting mash in small chick size hoppers.

At four weeks of age, feed fine oyster shell and grit on top of the mash in the regular hoppers.

At eight weeks of age, feed oyster shell and grit in special hoppers and keep them available to the turkeys at all times. Feed either turkey developer from eight weeks to maturity. If the poults are denied access to direct sunlight, cod liver oil should be continued in the developer mash until 10 weeks before the birds are to be marketed.

At 12 weeks of age, continue feeding the turkey developer and provide a grain mixture of 2 parts of cracked yellow corn and 1 part of wheat in open hoppers until the birds are marketed.

Summary

1. In the mash which was used as a basal ration in this study during the period from 1 to 13 weeks, the protein from 50 per cent of the dried milk and 50 per cent of either the meat meal or fish meal was satisfactorily replaced by either soybean oil meal or corn gluten meal, when the calcium and phosphorus losses were compensated.

2. In the ration fed from 13 weeks to maturity, satisfactory growth results were obtained when one-third or two-thirds of the total animal protein in the ration was replaced by an equal amount from soybean oil meal or corn gluten meal when the calcium and phosphorus losses were compensated.

3. Satisfactory growth was obtained when vegetable protein concentrates were substituted for a part of the animal protein concentrates in the starting and developing mashes.

4. Fourteen per cent soybean oil meal or 12.5 per cent corn gluten meal may replace equivalent amounts of animal protein in the 24 per cent turkey starter.

5. Corn gluten meal produced a superior fleshing condition.

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THE RELATIONSHIP OF THE VITAMIN D INTAKE OF THE HEN TO THE ANTIRACHITIC POTENCY OF THE EGGS PRODUCED^{1, 2}

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ONE FIGURE

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Hess ('23) found egg yolk to be one of the few important natural sources of vitamin D. Later, Hughes, Payne, Titus, and Moore ('25) conducted an investigation in which they fed egg yolks of known source to young chicks. These authors came to the conclusion that the vitamin D content of the egg depends on the diet of the hen, as well as on the light to which she is exposed. Bethke, Kennard, and Sassaman ('27) found that eggs from range hens which received direct sunlight were ten times as potent, antirachitically, as eggs produced by hens receiving the same basal diet, but which were confined indoors where they received only light that had been filtered through glass. These authors also found that 2 per cent of cod liver oil, when added to the basal diet, induced storage of five times as much vitamin D in the egg as did the basal diet alone or the basal diet supplemented by alfalfa ad libitum.

The investigations of DeVaney, Munsell, and Titus ('33) are the most recent reported in the available literature on this subject. These investigators made biological assays on

¹We wish to express our indebtedness to the National Oil Products Company, Harrison, New Jersey, for financial assistance in carrying out this investigation.

²Publication authorized by the director of the Pennsylvania Agricultural Experiment Station April 16, 1935, as technical paper no. 686.

egg yolks produced under controlled conditions, using young rats as the experimental animals. The technic employed is quite comparable to that in use at the present time. These investigators concluded that vitamin D from cod liver oil is more efficiently stored in the egg yolk than is vitamin D from an equivalent amount of viosterol, and that the vitamin D potency of egg yolk can be increased within certain limits by increasing the vitamin D intake of the hen.

Unfortunately, the data obtained in the investigations reported above cannot be compared on the basis of actual vitamin D unitage, owing to variations in the biological technic employed. It seemed desirable, therefore, that further efforts should be made toward securing additional information concerning the antirachitic potency of eggs produced under carefully controlled conditions. Since an investigation was already in progress at this institution which offered the opportunity of obtaining the eggs for such a study, it was highly desirable that an investigation be carried out at this time.

With the above facts in mind, the studies herein reported were initiated to determine the antirachitic potency of eggs produced by groups of hens which received diets varying only in their antirachitic potency.

EXPERIMENTAL

The eggs used in these studies were obtained from groups of hens which were being used in a series of nutritional studies in the department of poultry husbandry. These birds were denied access to sunlight and were maintained on a rachitogenic basal all-mash ration which was supplemented by varying amounts of vitamin D in the form of fortified cod liver oil, viz. (8D).³ Further information concerning the above investigation has been published elsewhere (Murphy, Hunter, and Knandel, '34).

The collection and the sampling of the eggs, and the preparation of the egg yolk samples were carried out in the following manner: The eggs were collected daily, weighed, labeled,

³This cod liver oil contained 100 Steenbock vitamin D units per gram, or approximately 8D potency.

and stored in a cool dark place until the end of the collection period. A complete record was kept for each bird in each of the several groups, in order that only representative eggs of each respective group might be used in making up the composite sample of yolks for biological assay. An effort was made to prepare composite samples of yolks, which represented an equal number of eggs obtained from the same number of birds in each of the respective groups.

Since only the yolks of the eggs were to be used in the biological assays, the eggs were broken and the yolks and the whites were carefully separated by hand. The selected yolks from each group of birds were weighed, thoroughly mixed, placed in sanitary cardboard containers, and stored at a temperature of -2° to -10°F. until needed in the feeding experiments.

The antirachitic potency of these samples was determined by means of biological assay, using young rats as the experimental animals. The rats used were produced in our own stock colony, and were 21 days of age and weighed approximately 40 gm. each when placed on experiment. These animals were kept in individual metal cages which were provided with raised screen bottoms and which were housed in a darkened room, from which all daylight was excluded. At the beginning of the experiment the rats were distributed among the several groups so as to eliminate possible variations that might arise as a result of litter or sex differences. Fresh distilled water and an ample supply of the Steenbock rachitogenic diet no. 2965 was kept before each animal at all times. After having received the rachitogenic diet for a period of 18 days, the test animals were found to manifest sufficiently severe rickets for assay purposes. In each case the degree of rickets manifested was verified by means of x-ray photographs.

In the first part of the investigation, the egg yolks to be tested were incorporated directly with a known weight of the antirachitic diet. It was found in subsequent assays, however, that more consistent results could be obtained by feeding the yolk separately, in three equal portions, on the first,

third, and seventh day of the curative period; consequently the latter procedure was adopted. In this procedure, the desired quantity of yolk was weighed directly into the small glazed receptacle in which it was fed. In a few cases where the quantity was too small to be weighed conveniently, an egg yolk-basal mixture was prepared in the ratio of 1:5 or 1:10, as conditions necessitated. The amount of the mixture which contained the desired amount of yolk was then weighed in three portions and fed in the usual manner.

During the 10-day curative period, the animals were weighed on the first, third, seventh, and tenth day. On the tenth day the animals were again x-rayed, killed, and 'line tested' in the usual manner.

The biological assays consisted of three parts, which were as follows:

a. Samples of egg yolks, obtained during the fall of 1933 from nine groups of yearling hens (hatched April, 1932) that were nearing the end of their first season of egg production, were assayed biologically in the above manner.

b. In order to obtain data concerning the effect of the period of egg production on the ability of the hen to transfer antirachitic potency from the diet to the egg, a second series of biological assays was conducted. The egg yolks used in this series were obtained during the spring of 1934 from eight groups of pullets (hatched November, 1933), which were receiving dietaries similar to those reported above, but which were just beginning their first season of egg production.

c. A third series of biological assays was made using egg yolks from three groups of yearling hens. One group received an optimum level of fortified cod liver oil, and the other two groups received excessive quantities of vitamin D in the form of fortified cod liver oil and viosterol (in corn oil), respectively.

The data obtained in this investigation are presented in condensed form in the following tables and figure (tables 1 to 3 and fig. 1).

In the case of the fall series, the sampling of eggs was begun on September 5th, and continued until September 15th. Since the hens used in this series were approaching the end of a season of egg production, the egg yield was irregular. This was especially true of those groups of birds which received less than the optimum amount of cod liver oil, and which were not allowed access to range. It was thought desirable that each sample be composed of a comparatively large number of egg yolks to insure a representative sample and to have sufficient material for feeding experiments. It was planned, therefore, to use the yolks from 3 dozen eggs in each sample, except in the cases of groups IV, VI, and VIII. Samples from these groups were to contain the yolks from a larger number of eggs, as these samples were to be used in the preliminary (trial) assays. However, as may be observed from table 1, such a systematic method of sampling was impossible owing to the fact that the hens comprising groups II and III produced so few eggs.

Table 1 also presents a summary of the results of the biological assays made of the nine samples of egg yolks produced by the respective groups of hens comprising the fall series. The percentage of positive line tests was computed on the basis of the number of animals which responded normally during the test period and which gave a two plus (2+)⁴ healing when line tested. When 60 per cent or more of the test animals showed a (2+) line test, after being fed a definite amount of the egg yolk, this amount of yolk was considered the critical level.

In the preliminary feeding technic, as has been previously stated, the weighed quantities of yolk were mixed with a known weight of the antirachitic diet and fed in this manner. Owing to the fact that some of the test animals used in the earlier studies died during the 10-day curative period, due in all probability to the partial decomposition of the yolk

⁴ An animal was rated as showing a (2+) healing when the line test showed a narrow continuous linear deposit of calcium salts on the metaphyseal side of the epiphyseal cartilage.

during the time it remained in the feed container, exposed to the warm temperature of the laboratory, the technic was modified so as to feed the yolk in small allotments, separately of the basal diet.

TABLE 1

Showing the results of the biological assays of the samples of egg yolks produced by groups of mature hens just before reaching the end of a period of egg production

GROUP NO.	PER CENT FORTIFIED COD LIVER OIL FED	NUMBER OF EGGS IN SAMPLE	WEIGHT OF EGGS IN GRAMS	PER CENT EGG YOLK	NUMBER OF RATS CONSIDERED	QUANTITY OF EGG YOLK FED GM./RAT/DAY	PER CENT POSITIVE LINE TESTS	PER CENT POSITIVE X-RAYS	CRITICAL LEVEL GM./RAT/DAY	UNITS OF VITAMIN D (STEEBOCK) PER GRAM OF EGG YOLK
II	1/32	14	711	33.3	6 5 6 2	0.7 0.8 0.9 1.0	70 80 80 100	0.7	0.14
III	1/16	10	557	32.1	2 5 5	0.5 0.6 0.7	0 20 60	0 20 60	0.7	0.14
IV	1/8	46	2459	32.7	2 6 5 6	0.1 0.2 0.3 0.4	0 0 20 75	0 0 0 33	0.4	0.25
V	3/16	36	1999	31.3	4 8 5	0.2 0.3 0.4	25 75 60	0 50 60	0.3	0.33
VI	1/4	59	3273	31.3	2 10	0.1 0.3	0 60	0 50	0.2	0.50
VII	3/8	36	2012	32.2	4 4 2	0.1 0.2 0.3	25 75 100	0 60 100	0.2	0.50
VIII	1/2	57	3113	32.3	10 1 1 1	0.1 0.2 0.3 0.4	60 100 100 100	40	0.1	1.00
IX	None (range hens)	35	1934	33.5	4 3 2 4	0.3 0.4 0.5 0.6	75 100 100 100	75 100 100 100	0.3	0.33
X	1/8 (range hens)	36	2011	31.4	4 7 2 3	0.025 0.05 0.1 0.2	50 71 100 100 100 100	0.05	2.00

The results presented in the table show that, in the case of the confined groups of birds, an increase in the amount of cod liver oil fed resulted in an increase in the antirachitic

TABLE 2

Showing the results of the biological assays of the samples of egg yolks produced by groups of pullets at the beginning of their initial period of egg production

GROUP NO.	PER CENT FORTIFIED COD LIVER OIL FED	NUMBER OF EGGS IN SAMPLE	WEIGHT OF EGGS IN GRAMS	PER CENT EGG YOLK	NUMBER OF RATS CONSIDERED	QUANTITY OF EGG YOLK FED GM./RAT/DAY	PER CENT POSITIVE LINE TESTS	PER CENT POSITIVE X-RAYS	CRITICAL LEVEL GM./RAT/DAY	UNITS OF VITAMIN D (STEEBOCK) PER GRAM OF EGG YOLK
II	1/16	36	1402	27.1	4 4 4	0.7 0.8 0.9	25 75 100	0 75 75	0.8	0.12
III	1/8	36	1506	30.3	4 4 4	0.4 0.5 0.6	0 75 100	0 50 100	0.5	0.20
IV	3/16	36	1502	27.1	4 4 4	0.3 0.4 0.5	25 75 75	25 50 75	0.4	0.25
V	1/4	36	1464	27.5	4 4 4	0.3 0.4 0.5	0 62.5 100	0 25 100	0.4	0.25
VI	5/16	36	1556	26.0	4 4 4	0.2 0.3 0.4	0 75 100	0 75 100	0.3	0.33
VII	3/8	36	1567	26.2	4 4 4	0.2 0.3 0.4	25 75 100	25 50 75	0.3	0.33
VIII	1/2	36	1536	27.5	4 4 4	0.1 0.2 0.3	0 75 100	0 75 100	0.2	0.50
IX	1	36	1560	25.9	4 2 4 2	0.05 0.075 0.1 0.2	62.5 75 87.5 100	25 50 75 75	0.05	2.00

potency of the eggs produced. When less than 1/8 of 1 per cent of the fortified cod liver oil was fed (e.g., groups II and III) egg production was so poor as to make it difficult to obtain representative samples of yolks for biological assays. In fact the results indicate that 1/16 of 1 per cent of the cod liver

oil was not better than $\frac{1}{32}$ of 1 per cent, in increasing the antirachitic potency of the egg yolks. It was interesting to note, however, that range hens which received no cod liver oil, but had access to sunlight (group IX) produced eggs equal in antirachitic potency to those produced by confined hens receiving the same diet supplemented with $\frac{3}{16}$ of 1 per cent of cod liver oil (group V). An addition of $\frac{1}{8}$ of 1 per cent

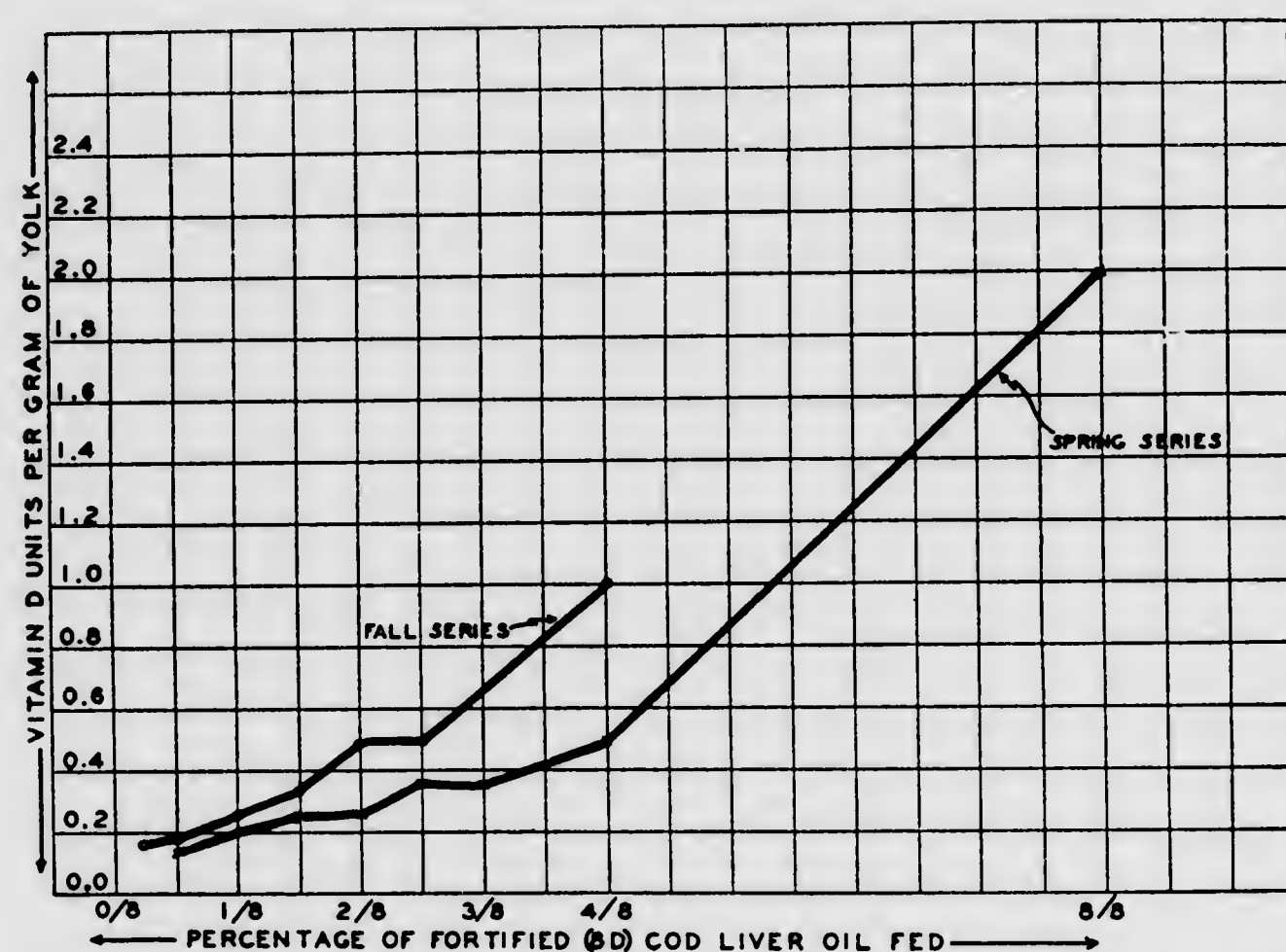


Fig. 1 Showing graphically the relationship between the percentage of fortified cod liver oil (8D) fed and the antirachitic potency of the eggs produced by the various groups of hens used in this investigation.

of cod liver oil to the diet of the range hens resulted in the production of eggs having approximately six times as much antirachitic potency as those produced by range hens receiving no cod liver oil (groups IX and X).

The relationship between the amounts of cod liver oil fed and the antirachitic potency of the eggs produced is presented graphically in figure 1.

Table 2 contains a summary of the data obtained in the second series of experiments. Since a systematic method of

sampling was possible in the case of this series, the samples consisted of two eggs from each of eighteen hens which were representative of the respective groups. It was necessary, however, to extend the collecting period for group II, 2 additional days, in order to obtain the desired number of eggs.

The vitamin D assay data found in this table were compiled in a manner similar to that described for table 1. Here, as in the first series, an increase in the amount of cod liver oil led to the production of eggs of higher antirachitic potency.

TABLE 3

Showing the results of the biological assays of the samples of egg yolks produced by the three groups of hens, two of which received excessive doses of vitamin D, as fortified cod liver oil and viosterol, respectively

GROUP NO.	PER CENT FORTIFIED COD LIVER OIL FED	NUMBER OF EGGS IN SAMPLE	WEIGHT OF EGGS IN GRAMS	PER CENT EGG YOLK	NUMBER OF RATS CONSIDERED	QUANTITY OF EGG YOLK FED GM. RAT DAY	PER CENT POSITIVE LINT TESTS	PER CENT POSITIVE X-RAYS	CRITICAL LEVEL GM. RAT DAY	UNITS OF VITAMIN D (STEELE-ROCK) PER GRAM OF EGG YOLK
I (control)	$\frac{1}{4}$ % SD oil	16	917	31.5	4	0.1	75	60		
					8	0.2	100	62	0.1	1.00
					8	0.3	88	88		
					4	0.4	100	100		
II (SD cod liver oil)	2 % SD oil	24	1299	30.8	4	0.015	25	25		
					7	0.025	70	57		
					4	0.035	100	100	0.025	4.00
					4	0.05	100	100		
					4	0.1	100	100		
III (viosterol)	2 % SD viosterol	9	497	32.1	4	0.015	0	0	0.025	2.00
					8	0.025	37.5	25	to	to
					4	0.05	100	100	0.05	4.00
					4	0.1	100	100		

The results of the biological assays were, in general, quite comparable to the results obtained through the fall series of assays. It is to be noted, however, that for a given level of cod liver oil the pullets at the beginning of a season of egg production produced eggs which were slightly less potent, antirachitically, than were eggs produced by yearling hens at the end of a season of production. But in this particular investigation, it appears that the number of eggs produced

might have been a more important factor in determining this difference in potency than the stage of egg production.

The relationship between the amount of cod liver oil fed and the antirachitic potency of the eggs produced by the various groups of pullets comprising the spring series is also presented graphically in figure 1.

The third phase of the investigation was concerned with the effect of feeding excessive quantities of vitamin D on the antirachitic potency of the eggs produced. Summaries of the collecting and the sampling of the eggs used in this series of assays and of the results of the biological assays are presented in table 3. The data given in this table were compiled in a manner similar to that described for the other series.

Considerable difficulty was experienced in obtaining sufficient eggs to comprise a representative sample from the group of hens which received the high intake of viosterol. This difficulty, however, was not encountered with the group of hens that received an equivalent amount of vitamin D in the form of fortified cod liver oil.

From table 3 it may be observed that when the antirachitic supplement was increased eightfold, by the addition of fortified cod liver oil, there resulted a fourfold increase in the antirachitic potency of the eggs produced (groups I and II). When the increased antirachitic supplement was fed in the form of viosterol (in corn oil), the antirachitic potency of the eggs produced was somewhat less than when an equivalent unitage of cod liver oil was fed.

Since the initiation of this investigation, there have appeared in print the publications of Branion ('34), Branion, Drake, and Tisdall ('34, '35), which likewise concern the vitamin D content of eggs. While the data herein presented do not permit a direct comparison with those presented by the above investigators (due to differences in methods of technic and in manner of presenting results), it appears that the data do indicate a somewhat lower antirachitic potency than might have been reasonably expected from the findings of the above investigators.

SUMMARY

A study has been made of the effect of the vitamin D intake of the hen on the antirachitic potency of the eggs produced. As a result of this investigation, the following conclusions are drawn:

1. The antirachitic potency of egg yolk depends on the antirachitic intake of the hen producing it.
2. The ability of the hen to transfer the antirachitic factor or factors from her diet to the egg appears to be limited.
3. When the hen's diet is supplemented with one-fourth per cent of fortified (8D) cod liver oil (which Murphy, Hunter, and Knandel ('34) found to be the optimum level when the birds were confined and denied access to direct sunlight), 1 gm. of the yolk from the eggs produced contained approximately 0.5 Steenbock unit. On this basis, an egg yolk weighing 15 gm. would contain 7.5 Steenbock units of vitamin D.
4. Yearling hens, nearing the end of a period of egg production, produced egg yolks that were more potent (antirachitically) than did pullets at the beginning of their initial period of egg production. It is suggested that this difference in potency might be due to the rate of egg production and not to the period of production.
5. At the particular level studied, the antirachitic potency of cod liver oil appeared to be more efficiently transferred from the diet to the egg yolk than was an equal unitage of viosterol.

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"That man is a benefactor to his race who makes two blades of grass to grow where but one grew before."

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Manganese and Some of Its Agricultural and Biological Relationships

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A GREAT deal of attention has been given to the subject of soil fertility. This involves many phases, including the mineral composition of the soil. Chief among these are studies relating to those elements which have long been known to be necessary for the growth and development of plants. Attention has been directed not only to the quantities of these elements present, and factors affecting their availability to the plant, but also to their utilization by animals which feed upon these plants. The elemental composition of the soil, therefore, is of vital importance to the nutrition of both plants and animals.

Until recently it was considered that ten elements would suffice for the growth of plants—carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, sulphur, calcium, magnesium, and iron. Lately, those elements which were found to occur in plants in smaller quantities and which had been considered as more or less "accidental constituents" of plants have been studied in their relation to the growth and development of living organisms. Several of these now are known to be necessary for plant growth, but in quantities much smaller than the more common plant nutrients, such as nitrogen, phosphorus, and potassium. Among these is the element *manganese*.

Some outstanding research work has been conducted on manganese in its relation to soil fertility and plant and animal growth. Some of the results will be set forth in this report.

No soil can be classed as fertile when manganese is lacking, or when it is present in an insoluble form. On the other hand, a soil may be infertile if it contains too large a quantity of water-soluble manganese. This is true of

other elements as well, even the more common ones, such as nitrogen, phosphorus, and potassium; but it is more difficult to render a soil less fertile with less active elements than the more active elements such as manganese or copper.

The Occurrence of Manganese in Soils

Agricultural soils vary widely in respect to their manganese content. Robinson (1914), in his study on the chemical composition of representative American soils, found that the content varied from zero to 0.31%, calculated as MnO, with an average of 0.071%. These results are more or less in keeping with those of Shedd (1914) in his work on Kentucky soils and with those of Carlyle (1931) in his work on Texas soils.

As a rule, the manganese content of soils is quite low. However, certain soils contain such large quantities of this element that they are alluded to as "manganiferous soils." Kelley (1912), in his work on Hawaiian soils, reported an instance where the manganese content, calculated as Mn₂O₄, was equal to 9.74% of the soil mass.

On the whole, there appears to be more manganese in productive than in unproductive soils, and more in heavy than in light soils. Meyer (1932) found more of this element in virgin than in cultivated soils, which is to be expected.

The Availability of Soil Manganese

Some of the soil manganese is found in true solution and some in replaceable form. These are the sources of manganese for plant growth. According to Schollenberger and Dreiblebis (1930) the replaceable manganese may be re-

duced to a minimum through cropping and liming the soil.

Most of the soil manganese, according to Piper (1931), usually occurs in a highly oxidized and insoluble form. This may be rendered more soluble on reduction through the addition of organic matter or by steam sterilization. It may be reduced by the exclusion of air, as when a soil contains excess water.

The principal factor involved in the availability or non-availability of manganese is the reaction of the soil itself; it is more available under acid than under neutral or alkaline conditions. According to Bear (1929), any soil having an alkaline reaction may show a deficiency of manganese.

The work of many other investigators leads to the same conclusions. Skinner and Ruprecht (1930) report that on certain Florida soils, normal plant growth can be obtained only by fertilization with manganese salts. These soils are underlain with a layer of calcium carbonate and the reaction of the soils varies from a pH of 7.5 to a pH of 8.8.

While some soils are inherently alkaline, others may be rendered so through the injudicious use of lime. In either case, one must expect the manganese to be present in an insoluble and unavailable form. Such soils would benefit through fertilization with manganese salts, keeping in mind, however, that more care should be taken in regard to the quantity applied than is true for the ordinary fertilizing materials.

If the soil contains a normal quantity of manganese and is slightly acid in reaction, sufficient quantities of this element should be rendered soluble and available for plant growth. However, when the soil contains very small quantities of this element, according to Willis (1932), manganese may be a limiting factor even though the soil is slightly acid in reaction. Connor (1932) states that, while certain Indiana soils appear to have sufficient quantities of manganese present for normal crop production, the points at which it is either available or unavailable are so close that different crops, different fertilizers, and different climatic conditions may make this element present in sufficient quantity or deficient.

Manganese Fertilization

When the need for manganese has been fully established, a manganese salt, usually the sulphate, has been added to the soil as a fertilizer, or to the crops in the form of a spray. If an addition of manganese sulphate is deemed necessary, the quantity to be added must be gov-

erned by the requirements of both the soil and the crop in question.

Skinner and Ruprecht (1930) have used from 50 to 150 pounds per acre with striking results on Florida glade soils. On the other hand, Gilbert and McLean (1928) found that 8 pounds of manganese sulphate per acre was sufficient to correct this deficiency on certain Rhode Island soils, while Hartwell (1926) on somewhat similar soils found that 80 pounds per acre was beneficial if the soils were neutral to alkaline in reaction, and that 40 pounds constituted an excess when the soils were acid.

As a rule, the beneficial effects of manganese may be attributed to its use as plant nutrient. However, it may be indirect as well as direct in effect. Brown and Minges (1916) found that manganese fertilization was beneficial to the process of nitrification, while Deatrick (1918) found that such additions are beneficial to ammonification.

Kelley (1914) found that the addition of manganese salts as fertilizers resulted in a marked increase in the availability of both calcium and magnesium. He attributed the stimulatory effect of manganese addition on crop growth to this effect. On the other hand, Schreiner and Sullivan (1910) found that manganese additions promoted soil oxidation. Later Schreiner and Reid (1912) substantiated these results and further established a more or less direct relationship between the oxidative power of a soil and its productivity.

Relation of Manganese to Plant Growth

Many investigators since the time of Scheele (1785), who found manganese in the ash of the "wild anise," have been concerned with the occurrence of manganese in the plant kingdom. The results of their studies show that it is present in all plants. Its necessity for plant growth, however, was not recognized until quite recently. Bertrand (1910, 1912) probably was the first to call attention to the fact that it is indispensable, while McHargue (1922) fully established the fact that plant growth is impossible in its absence.

The Manganese Content of Plants

While manganese is universally distributed throughout the plant kingdom, the quantity varies according to the kind of plant, its age and the conditions under which it is grown. According to Robinson (1917) the percentage fluctuates more than for any other mineral element.

Schroeder (1878) found that the ash of certain pine needles contained 35.53% of manganese, calculated as Mn_3O_4 , while the ash of

pine bark contained as high as 41.23%. Hilgard (1860) likewise reports a very high percentage in the ash of Mississippi long-leaf pine.

A high percentage of manganese in plants is the exception rather than the rule. Peterson and Lindow (1928) found that ordinary crop plants contained but small quantities of this element as compared to other elements present.

McLean and Hardin (1926) found a variation of from 5 to 290 parts per million of manganese in oats, based on the weight of dry matter.

Jacobson and Swanback (1932) found that while tobacco may be looked upon as a "luxury feeder" of manganese, yet its growth can be depressed if a certain concentration is exceeded. This danger may be encountered with the majority of other plants. On the other hand, Bolin (1934) has shown that the manganese content of alfalfa is not only low but also that it does not fluctuate to any great extent. Incidentally, this low manganese requirement may account, to a certain extent, for the good growth of alfalfa on a non-acid soil.

This wide variation in the manganese content of plants, which is greater than that of any other element, renders it difficult to calculate the manganese requirements of plants and calls for experimentation on the plant and locality in question.

Localization of Manganese Within the Plant

Jardin and Astruc (1913) found manganese localized in the actively growing portions of the plant, a fact which has been fully established by many others. In all cases, the leaves contain far more than the stalks.

McHargue (1914) found manganese in greater concentration in the outer membranes of wheat grains and least in the starchy portion. In the manufacture of patent flour, therefore, most of the manganese would be lost in the waste. For this reason, graham bread is better than white bread as a source of manganese. On the other hand, the manganese content of the oat grain is more evenly distributed. According to McHargue, oatmeal constitutes one of the best sources of manganese in the diet.

Role of Manganese in Plant Growth

The role of manganese in plant growth is rather obscure. McHargue (1922) believes that this element is concerned with the synthesis of proteins and carbohydrates as well, but further work is desirable before this can be fully established. Other points, however, have been quite well established.

McHargue (1922) considers that manganese, with iron, aids in the synthesis of chlorophyll. Chapman (1931) established the fact that manganese aids in the transport of iron and it is well known that under normal conditions iron is necessary for the synthesis of chlorophyll. A greener color of manganese-treated over control plants was observed by Meyer (1932); this would tend to substantiate the contentions of McHargue.

Relation of Manganese to Biological Oxidations

Bertrand (1897, 1905, 1912) considers that the addition of manganese to growing plants increases the oxygen-carrying power of oxidase enzymes. Deatrick (1919) found that such additions seemed to increase the oxidizing power of the roots of wheat seedlings. This was later established by Eversmann and Abernethy (1927). McHargue (1914) found that manganese was localized in those areas of plants which had to do with the secretion of oxidizing enzymes. His inference is that manganese serves as a catalyst for these enzymes.

Hopkins (1930) states that the function of manganese may be one of activation of iron in the plant. The hypothesis is that iron is reduced in the organism, a point that is fully established, and that manganese serves to reoxidize it. However, other mechanisms have this power, and manganese is required in addition, so that this role of manganese may not be vital to the welfare of the plants.

Relation of Manganese to Disease Resistance

Samuel and Piper (1928) report that a condition known as the "grey-speck" disease of oats is not a true disease but a condition brought about by lack of available manganese in the soils on which the plants are grown. This condition can be rectified by spraying the plants with solutions of manganese salts. Teakle (1933) reports a condition formerly recognized as a disease of oats and barley called "Roadside-take-all," a condition which he associated with lack of available manganese in the soil and which could be overcome by manganese additions.

In general, any plants containing the necessary mineral elements in proper proportions are more resistant to disease than plants containing too much or too little of any essential element.

Symptoms of Manganese Deficiency

Gilbert (1934) states that lack of sufficient quantities of manganese produces a yellowing or reddening of plants, which he called "chloro-

sis". The symptoms vary with different plants, due to the prevalence of green, red or purple pigments. With spinach, the normal green color changes to a pale yellow or to a golden yellow. Similar plants show identical symptoms.

With table beets the symptoms are somewhat different. Instead of becoming progressively yellow, the leaf gradually takes on a deep red to purple color.

Under more pronounced conditions, according to Samuel and Piper (1929), manganese deficiency results in arrested development, followed by death of undeveloped tissue at the growing point.

Symptoms of Excess Manganese

Considerable difficulty has been encountered in obtaining normal plant growth on manganiferous soils. McGeorge (1923) found that pineapples grown on these soils developed a condition of chlorosis, which he attributed to a greater assimilation of calcium than on a normal soil. This, he stated, would result in a precipitation of iron in the cells. Such a condition could be rectified by spraying the plants with a 10 per cent solution of ferrous sulphate.

Kelley (1914) states that pineapples grown on highly manganiferous soils showed a woody root system, and a gradual yellowing of the leaves followed by the development of yellow spots which spread over the entire leaf. Such plants, as a rule, produced very small fruits, abnormally pink in color.

These experiments suggest that excess of manganese is undesirable. McCool (1934) found that in certain cases the steaming of soils resulted in an increased concentration of soluble manganese, sufficient to seriously interfere with plant growth. Such a condition, however, could be overcome by adding superphosphate which brought about the formation of the less soluble manganese phosphate.

What holds true for manganese, likewise holds true for other essential elements; an excess is almost as undesirable as a deficiency.

Relation of Manganese to Animal Growth

Manganese is as widely distributed throughout the animal kingdom as the plant kingdom. It is found in all tissues but the concentration varies according to the tissue. According to Swartz (1929, 1930) it occurs in larger quantities in the liver, pancreas, lymph nodes and kidney than in other animal parts.

Until recently, manganese was not considered essential to the animal diet, but now it is recognized to be necessary.

Absence of manganese from the diet of female rats brings about a disturbance of the oestrus cycle and likewise a failure of the development of the mammary glands, making it impossible for the mothers to suckle their young. In male rats, complete degeneration of the germinal epithelium occurs, according to Bodansky (1934).

Titus, Cave and Hughes (1928) found, in support of the work of others, that iron could be utilized for the building of hemoglobin in the animal body if copper was likewise present. However, the presence of manganese was found to be extremely desirable, if not essential, for this process to be effected.

If manganese is necessary for animal growth and development, the question of the manganese content of plants should be considered from this standpoint as well as from the standpoint of the growth and development of the plants in question. Hence, the relation of manganese to soil fertility, plant and animal growth, assumes an added significance.

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[CONTRIBUTION FROM THE LABORATORY OF THE DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL CHEMISTRY, THE PENNSYLVANIA STATE COLLEGE]

Ricinus Lipase, its Nature and Specificity¹

BY HERBERT E. LONGENECKER² AND D. E. HALEY

The lipolytic principle of the castor bean (*Ricinus communis*) has engaged the attention of many research workers since its presence was first proved in 1890 almost simultaneously by Green³ and Sigmund.⁴ However, a review of the available literature on the active substance, Ricinus lipase, has failed to reveal any general concordance of experimental results. Haley and Lyman's discovery⁵ that with oils optimum conditions for activity were obtained only within narrow limits of hydrogen ion concentration, about pH 4.7-4.8, made it necessary to repeat most of the previous work.

The object of the present investigation was to prepare a highly active, dry and stable preparation of Ricinus lipase from resting castor seeds and to study the nature of its action and its relative specificity in the hydrolysis of various vegetable and animal oils. These studies were undertaken to elucidate the mode of action of Ricinus lipase and to contribute to the knowledge of enzyme action in general.

Experimental

A. Preparation of Lipase Material.—Ricinus lipase was prepared from large size castor beans⁶ by hulling and

macerating them, extracting the fat as completely as possible with low boiling petroleum ether (20-40°), and finally drying, pulverizing and sifting the finished product through a 60-mesh sieve. This is a modification of the method given by Haley and Lyman.⁵ Other methods for the preparation of Ricinus lipase have been reviewed by Longenecker.⁷ Most of them are less suitable.

B. Estimation of Activity.—In all experiments reported here, a standard procedure was adopted for the determination of lipolytic activity: 1.00 gram of oil was weighed into five-cc. hard-glass bottles and two drops of toluene added to prevent bacterial action. To this was added a weighed quantity of enzyme material and its activator, tenth normal acetic acid. The pH of the resulting mixture was 4.8. It was emulsified by vigorous shaking for three minutes. Digestion was carried on at a temperature of 37°. To prevent loss of any of the digestion materials, the bottles were stoppered with paraffined corks. The results reported represent averages of triplicate determinations. "Blanks" always accompanied the samples.

After a definite time of incubation, the reaction mixture was placed in 50 cc. of hot 95% alcohol and the free fatty acids in the digestion mixture were titrated with 0.1 N alkali using phenolphthalein as the indicator. The starting time for each experiment was that time when acetic acid was added to the digestion mixture; the final time, when the reaction mixture was placed in the alcohol. It was found by preliminary experiments that no hydrolysis took place until after addition of the acid and that hydrolysis was completely inhibited in the amount of alcohol used in the titration.

Titration results are of little value, *per se*, in studies of the relative specificity of lipolytic catalysis of oils although several workers have reported their findings in this way. A more significant expression is the percentage of oil hydrolyzed or the number of moles of triglyceride hy-

(7) Longenecker, M. S. Thesis, The Pennsylvania State College, 1934.

(1) Presented before the Division of Biological Chemistry at the 90th meeting of the American Chemical Society, San Francisco, California, August 19-23, 1935.

(2) Submitted in partial fulfillment of the requirements for the degree of Master of Science at The Pennsylvania State College.

(3) Green, *Proc. Roy. Soc. (London)*, **48B**, 370 (1890).

(4) Sigmund, *Monatsh.*, **11**, 272 (1890).

(5) Haley and Lyman, *THIS JOURNAL*, **43**, 2664 (1921).

(6) Courtesy of The Baker Castor Oil Co., Newark, N. J.

drolyzed. For the calculation of the percentage hydrolysis, the formulas suggested by Sudborough, Watson and Varma and Wilson and Merrill⁸ have been used as a basis. The difference between the volume of alkali required for the sample and that required for the blank is a measure of the fatty acids resulting from the direct cleavage of glycerides due to the action of the lipase; the ester number is a measure of the total fatty acids in glyceride combination. To obtain the percentage hydrolysis, the following formula has been used

$$\frac{\text{cc. 0.1 N alkali (sample)} - \text{cc. 0.1 N alkali (blank)}}{\text{Saponification no.}^a - \text{Free fatty acid no.}^a} = \% \text{ hydrolysis}$$

^a Expressed in cc. of 0.1 N alkali.

C. Activity of Old and New Preparations.—The ability of Ricinus lipase to retain its activity over a long period was demonstrated in the following experiment in which the activity of a sample similarly prepared by one of us in 1924 was compared with that of a freshly prepared sample: 1.00 gram of olive oil and 0.6 cc. of 0.1 N acetic acid were used; the amount of enzyme material was varied; one set of samples (A) was shaken for three minutes at the start, then allowed to stand for twenty-four hours at 29–30°; another set of samples (B) was shaken continuously by machine for fourteen hours then allowed to stand for ten hours at 29–30°. The results are given in Table I.

TABLE I
COMPARISON OF ACTIVITIES OF TEN-YEAR OLD AND NEW PREPARATIONS

Treatment	Percentage hydrolysis		Hydrolysis new sample	
	ten-year old sample 0.050 g.	0.100 g.	0.050 g.	0.100 g.
A	6.79	17.82	77.83	86.00
B	14.03	49.36	85.43	86.70

TABLE II
TYPICAL RATE OF REACTION AND THE EFFECT OF TEMPERATURE AND SHAKING

In each experiment, 1.00 g. of olive oil, 0.6 cc. of 0.1 N acetic acid, 0.100 g. of lipase material and two drops of toluene were shaken together and incubated at 37–38°. A, shaken for three minutes at the start. B, shaken for three minutes at the start, let stand for thirty minutes and shaken again for two minutes. C, same as A except that temperature was 27–28°.

Time of digestion	Percentage hydrolysis		
	A	B	C
5 min.	11.02
10 min.	19.35	...	25.88
15 min.	31.91
20 min.	38.33	...	44.70
30 min.	39.22
40 min.	50.63	...	55.88
45 min.	...	57.93	...
50 min.	51.91
60 min.	59.60	66.39	59.66
2 hrs.	65.37	74.85	67.80
3 hrs.	71.14	79.34	71.90
5 hrs.	72.42	80.50	...
10 hrs.	74.21
24 hrs.	77.80	77.42	...

(8) Sudborough, Watson and Varma, *J. Indian Inst. Sci.*, **2**, 241 (1919); Wilson and Merrill, *J. Am. Leather Chem. Assoc.*, **1** (1926).

D. Rate of Hydrolysis of Olive Oil.—Olive oil was selected as a substrate to demonstrate, first, typical rates of reaction and second, some of the factors influencing it. In Table II, data are given which express (A) a normal rate of reaction, (B) the effect of shaking the digestion mixture after the reaction has proceeded for thirty minutes and (C) the effect of temperature.

E. Relative Specificity of Ricinus Lipase Action.—A study of the rates of hydrolysis of various members of a general group of substances should yield information as to the relative specificity or affinity of an enzyme for a particular substrate. A variety of oils of vegetable and animal origin were selected for specificity studies. Rates of hydrolysis of these oils are found in Table III.

Discussion

In analyzing and interpreting these results the nature of the active material must be considered. "Ricinus lipase" has been used here to represent the hulled, fat-free, resting castor seeds. Proteins, carbohydrates and salts originally present have not been removed from the final product.

It is significant that a ten-year old preparation retained a fairly high activity compared with a fresh sample. Enzymatic activity is known to decrease considerably in short periods, especially when the enzymic material is in solution. Dry preparations are usually more stable. So far as the authors can ascertain, however, there is no report in the literature showing the retention of activity of any enzyme preparation over a period of ten years.

One condition that appears to be a *sine qua non* factor for Ricinus lipase activity is the colloidal state. One may assume that an emulsion arises in a heterogeneous system such as the digestion mixture of oil, enzyme and dilute acid. Sudan III and conductivity tests indicated in this work that it is of the water-in-oil type under the experimental conditions. This type of emulsion was favorable for the action of Ricinus lipase. In fact, there is every reason to believe that the enzyme is not as active in an emulsion of the opposite type.

The production of a suitable emulsion was aided decidedly by shaking the digestion mixture. Shaking by hand for three minutes ensured that approximately the same degree of emulsification was obtained at the beginning of each experiment. Once formed, however, the emulsion was not stable. It tended to separate into layers of water and oil within one-half hour, when the rate of hydrolysis began to decrease. Shaking the digestion mixture at this point restored the emul-

TABLE III

RATES OF HYDROLYSIS OF VARIOUS OILS OF VEGETABLE AND ANIMAL ORIGIN

In each experiment, 1.00 g. of oil, 0.100 g. of lipase material, two drops of toluene and 0.6 cc. of 0.1 N acetic acid were shaken together for three minutes at the start, then let stand at 37–38°.

Oil	Minutes					Hours			
	15	30	45	60	120	3	5	10	24
Castor	33.33	47.48	59.15	59.15	72.70	83.30
Coconut	30.67	43.26	48.96	50.73	54.27	59.58	61.37	65.81	67.05
Corn	35.37	53.86	60.74	67.16	71.64	72.09	81.79	87.01	90.75
Cottonseed	36.96	52.90	62.75	63.33	72.61	71.30	83.33	86.52	87.25
Fish	17.66	22.24	26.81	27.33	31.48	33.24	36.06	37.00	41.67
Linseed	21.44	34.25	38.78	42.88	52.48	56.61	71.12	72.59	72.90
Neat's-foot	28.93	32.43	43.11	47.96	57.86	63.69	68.31	69.33	70.68
Olive	31.91	39.22	50.63	59.60	65.31	71.14	72.42	74.21	77.80
Peach kernel	24.36	33.35	47.57	52.20	57.57	61.20	65.54	70.18	69.60
Peanut	37.55	55.86	63.55	67.40	77.73	83.70	94.40	97.24	...
Rape	39.52	48.36	54.95	62.02	71.49	69.89	77.44	81.14	83.45
Sperm	10.18	13.71	16.83	20.36	18.28	18.89	23.68	19.53	23.47
Soybean	17.66	35.05	53.13	61.30	77.64	73.99	84.35	84.94	89.17
Whale	16.77	25.33	26.26	28.98	32.82	35.14	37.67	41.81	41.71

sion and with it there was an increased rate and extent of reaction in a given time, as is seen in Table II.

A higher percentage of hydrolysis in cases where a lower temperature was used, as shown also in Table II, can be correlated with the stability of the emulsion. In each case it was observed that the tendency for the emulsion to break was much less at the lower temperature.

That Ricinus lipase exhibits a differential rate of attack for various oils appears obvious from Table III. Previous investigators have reported similar experiments and results.^{9–13} In the majority of cases, it has been emphasized that the Ricinus lipase exhibited a relative specificity for those glycerides of high molecular weight. The results above seem to offer support for this argument, with a few exceptions. Thus, for the various oils used, listed in the order of decreasing percentage hydrolysis, the mean molecular weights are:

Oils	Mean mol. wt.	Oils	Mean mol. wt.
Peanut	870	Linseed	786
Castor	921	Neat's-foot	995
Corn	895	Peach kernel	785
Cottonseed	870	Coconut	589
Soybean	869	Whale	590
Rape	956	Fish	616
Olive	765		

However, if another line of reasoning be adopted, this apparent specificity disappears almost entirely. When the number of moles of

(9) Counstein, *Ergebnisse der Physiol.*, **3**, 194 (1904).

(10) Tanaka, *J. Coll. Eng. (Imp. Univ. Tokyo)*, **5**, 25, 152 (1912).

(11) Takamiya, *J. Agr. Chem. Soc. (Japan)*, **5**, 595 (1929).

(12) Falk, *This Journal*, **35**, 616 (1913).

(13) Sudborough and Watson, *J. Indian Inst. Sci.*, **5**, 119 (1922).

glyceride hydrolyzed after a given time is considered, no relative specificity of Ricinus lipase is observed. The results are all of the same order of magnitude and the probability that any individual variations are due to an actual difference in specificity is very low indeed.

Summary and Conclusions

A dry, stable and highly active preparation of Ricinus lipase has been obtained from hulled castor beans by extracting the fat with low boiling petroleum ether, pulverizing and sifting the residue of the bean through a 60-mesh sieve. The activity of this fresh preparation was compared with that of one ten years old. Considerable activity was obtained with the latter.

A water-in-oil emulsion appears to be desirable for Ricinus lipase action. The formation of this emulsion was aided by shaking and by lower temperatures of incubation.

Rates of hydrolysis of a variety of oils of vegetable and animal origin were obtained for studies on the nature of action and specificity of Ricinus lipase. Under the experimental conditions, Ricinus lipase catalyzed the hydrolysis of the following oils, listed in order of decreasing percentage hydrolysis after a given time: peanut, castor, corn, cottonseed, soybean, rape, olive, linseed, neat's-foot, peach kernel, coconut, whale, fish and sperm. An analysis of the data on the basis of the number of moles hydrolyzed revealed that the lipase showed no specificity in its attack on glyceride molecules containing carbon chains of different length.

STATE COLLEGE, PENNA.

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FOODS WE EAT AND WHY WE EAT THEM

By Professor R. ADAMS DUTCHER

DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL CHEMISTRY, PENNSYLVANIA STATE COLLEGE

At the Pennsylvania State College we have a student dining room which the architect has designed as a Pennsylvania Dutch grill. Over the counter from which the foods are served there is inscribed in German script the Pennsylvania Dutch motto, "Sak mir was du esscht und Ich sak dir was du bisseht." This is the Pennsylvania Dutch equivalent for the old maxim, "Tell me what you eat and I will tell you what you are." A recent writer has paraphrased this maxim by saying, "Tell me what you eat and I'll tell you how old you are." He informs us that it is possible to divide the span of human life into eleven dietary or gastronomic ages, which fall in the following chronological order:

- Age No. 1—Milk.
 " " 2—Bread and milk.
 " " 3—Milk, eggs, bread and spinach.
 " " 4—Oat meal, bread and butter, green apples and all day suckers.
 " " 5—Ice cream soda and hot dogs.
 " " 6—Minute steak, fried potatoes, coffee and apple pie.
 " " 7—Bouillon, roast duck, scalloped potatoes, French rolls, creamed broccoli, fruit salad, divinity fudge and demi tasse.
 " " 8—Pate de foies gras, wiener schnitzel, potatoes parisienne, egg

plant a l'opera, demi tasse and Roquefort cheese.

- " " 9—Soft boiled eggs, toast and milk.
 " " 10—Crackers and milk.
 " " 11—Milk.

While it is clear that this writer was poking fun at our dietary inconsistencies, he was also emphasizing a very important dietary truth, *viz.*, that milk plays a very important part in giving us the proper start in life and that we instinctively revert to milk when we have reached the age when our digestive organs begin to show the results of unwise dietary excesses of former years.

Our knowledge of foods and food values has undergone many changes as a result of chemical and nutritional researches. Hippocrates (460-370 B.C.) believed that there were many kinds of food but that there was but one single substance in foods which was necessary for normal growth and development. As late as 1813 the eminent French physiologist, Richerand, still adhered to this hypothesis. Twenty years later (1833) Dr. William Beaumont published his classical work on gastric digestion and at that late date he referred to the nutritive value of foods in terms of a

single substance which he called "aliment." It would be very easy to solve our dietary problems if this theory could be shown to be correct. Unfortunately for the layman, each new discovery in food chemistry seems to make the problem more complex.

Dr. William Prout, an English physician, was one of the first scientists to "complicate the picture" when he announced in 1834 that foods contain at least three essential types of materials which are necessary for good nutrition. These substances were called albuminosa, oleosa and saccharosa, which are known to us as proteins, fats and carbohydrates.

Justus von Liebig, of Germany, was largely responsible for the conception that fats and carbohydrates are used largely as sources of heat and energy in the animal body, while proteins function primarily as building materials for body tissues.

As chemistry developed, foods were classified according to their content of these three substances, and the first feeding standards for humans and domestic animals were, naturally, chemical standards. The first period, therefore, might well be called the "chemical period" in nutrition. This was followed by a second period, which might be called the "energy period," in which food values were expressed in terms of calories or heat units. It was not long, however, before it became evident that diets could be compounded from chemically purified proteins, fats, carbohydrates and mineral salts, which conformed to the best of the chemical and energy feeding standards and yet lacked something necessary for normal growth and reproduction.

A new method of research was devised, using chemically purified diets and small experimental animals, with the result that a new (biological response) period came into existence. This period of research has led to what is popularly called the "Newer Knowledge of Nutrition." During this period three important types of discoveries were made, which have had

considerable influence on the food habits of the American people.

Prior to this period chemists and nutritionists believed that all proteins, regardless of source, possessed the same nutritive value because they all contained about the same amount of nitrogen. The new work with small animals proved that this is not the case, since animals grow better on some proteins than they do on others. Subsequent investigations by the ever curious research chemist led to the discovery that proteins of high biological value contained many amino-acids (which act as building stones for body tissues), while the poorer proteins were found to be lacking in some of these amino-acids which were needed by the body tissues.

When the missing amino-acids were added to the inferior proteins or when other proteins were added to the diet, rats again grew normally. Thus, the first important lesson to be learned is that, as a class, individual animal proteins are nutritionally superior to individual vegetable proteins and that the wise mother will plan her meals in such a manner that her growing children will be certain to receive all the amino-acids necessary for the construction and repair of muscles and similar tissues. This can be done by supplementing the important energy-producing foods, such as bread and cereals with vegetables, milk, eggs and meat.

The second discovery that resulted from work with small animals had to do with the mineral salts in the diet. Scientists already knew, of course, that the growing child and even mature people must have mineral salts in order to construct strong bones and teeth and keep the blood and body fluids in healthy condition. They had the feeling, however, that the mineral problem "sort of took care of itself" if the other ingredients, such as proteins, fats and carbohydrates, were provided in ample amounts.

The biological response tests with small animals soon exploded this idea, for it

was found that some of our daily diets were woefully lacking in certain mineral or inorganic salts. This type of research also led to a better understanding of the individual functions of the common inorganic elements, such as calcium, phosphorus, magnesium, sodium, iron and chlorine. Much to our surprise we began to discover that some of the rarer elements, such as copper, iodine, manganese and even zinc, might be vitally essential for health and well-being. In former years copper was considered a member of the "poison family"—to be gastronomically avoided. Biological tests have shown, however, that copper in traces has a very beneficial effect on the utilization of iron and helps us to build better red blood cells.

It is entirely possible that we may find eventually that practically all the inorganic elements have a definite function to perform, in spite of the fact that they are present in almost infinitesimal traces. Iodine is a case in point. When it is absent from the soil, food crops and waters—such as we find in goitrous regions—excellent prophylactic results can be obtained by eating foods or drinking water containing very small quantities of iodine. Again the conclusion seems inevitable—that we are more likely to obtain the mineral salts we need, if we will vary our diet sufficiently to ensure a varied supply of mineral elements.

I have already pointed out that milk contains proteins of high nutritive value. Milk also contains a very desirable mixture of inorganic salts. In fact, milk is our best single source of calcium, and research has shown that it is quite difficult for the growing child to obtain its daily calcium requirement unless it receives from a pint to a quart of milk daily. It is not necessary to drink this amount of liquid milk to obtain this amount of calcium. It is possible to ingest a portion of the milk as dried or evaporated milk in cooked foods, puddings, etc. The vegetables, particularly the leafy vegetables, also contribute ap-

preciable amounts of calcium and other valuable mineral elements, such as iron. Thus we have learned to drink more milk and eat more vegetables and fruits in order that our mineral salt supply may be adequate for normal health and well-being.

A third discovery that developed from animal experimentation was even more unexpected and unbelievable, *viz.*, the discovery of vitamins. It was found that some foods did not contain these mysterious chemical substances and if these foods were fed for long periods of time (without supplementation with other foods) animals ceased to grow, failed to reproduce and developed disease symptoms identical with or similar to human diseases that have been known by the medical profession for centuries. It was soon possible to produce experimental dietary diseases similar to if not identical with human beriberi, pellagra, rickets and scurvy. Then discoveries came so fast that even the research workers themselves had difficulty keeping up with the newest developments.

Vitamin A received considerable front page publicity because its absence from the diet caused loss of weight, respiratory diseases and blindness in rats, which could be prevented when milk, butter fat, egg yolk, fish liver oils, carrots or pigmented leafy plants were added to the diet. The world war presented an opportunity to test the value of rat experiments on humans because children in Rumania and Denmark were deprived of dairy products which resulted in pathological eye symptoms similar to those produced in vitamin A-deficient rats. When Denmark discovered that she was selling her milk and butter at the expense of the health of her children, she eliminated the eye disease by regulating the amount of dairy products that could be shipped from the country. In Rumania the problem was solved by administering cod liver oil to all children.

Brilliant researches of the past few years have shown that the yellow pig-

ment carotene, found in carrots and many plants, can be changed into vitamin A by the body tissues. The cow takes this pigment from her feed, changes a portion of it into colorless vitamin A and excretes the excess of yellow pigment in the milk, where it is available for us to transform it to vitamin A in our own tissues. Guernsey and Jersey cows can not change this pigment into colorless vitamin A quite so readily as the Holstein and Ayrshire breeds, with the result that butter fat from the former is more yellow than that from the latter, but the total biological values (from the standpoint of carotene and vitamin A) are equal, the former being richer in the provitamin, carotene, but poorer in colorless vitamin A. The reverse is true for Holstein and Ayrshire butter fats, since they contain more of the colorless vitamin A but less of the yellow carotene. Since Guernsey and Jersey milks usually contain higher percentages of butter fat, the total vitamin A effect of the milk may exceed that of the non-pigmented breeds. These are no longer mysterious substances, for we are now able to write the chemical formulae for vitamin A and for carotene.

Vitamin B or B₁ (anti-beriberi vitamin) has now been crystallized and its chemical formula is practically assured. This vitamin is necessary for normal health, appetite and growth and is found in cereals, yeast, milk, leafy vegetables and fruits.

Vitamin C is now being manufactured artificially by the pound, its chemical structure has been determined and the chemist calls it ascorbic acid. When we eat liberal amounts of oranges, lemons, tomatoes, fresh cabbage, lettuce and similar foods we are certain to obtain sufficient quantities of this scurvy-preventing vitamin.

An artificial type of vitamin D (known as activated ergosterol) has also been crystallized. It has been obtained in

pure form and its chemical formula is known with a fair degree of certainty. This rickets-preventing vitamin is necessary for the proper building of bones and teeth in growing children. Our best natural sources are the fish liver oils. Milk does not contain it in satisfactory amounts, as a rule, which accounts for the fact that we find various types of vitamin D milk on sale which have been supplemented with various forms of vitamin D.

Vitamin E, the fertility or anti-sterility factor, will not be discussed because its clinical importance has not yet been fully established.

Vitamin G or B₂ promotes appetite and well-being. Without it a number of pathological conditions develop of which a pellagra-like skin disease is the most outstanding. We find this vitamin in many foods, such as yeast, whole cereals, milk, vegetables and fruits. Investigations of the past few months indicate that vitamin G (B₂) consists of two separate substances. One of these is a yellow pigment called "flavine" which is thought to be necessary for growth. The other fraction, tentatively called vitamin B₆, is thought to be specific in preventing and curing a dietary dermatitis in rats which is similar to pellagra in human beings. A number of other "B vitamins" have been suggested, but we have no information to date that they have any importance in human nutrition.

The newer knowledge of nutrition has taught us to be reasonably careful to supplement our daily diet of meat, bread and potatoes with the "protective foods," such as milk, eggs, fruits and vegetables. It is unwise and unscientific to condemn wholesome foods merely because they are lacking in certain dietary essentials. It is much safer and more economical from a health standpoint to supplement such foods with reasonable amounts of milk, eggs, vegetables and fruits.

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